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(54) Title: ALLOSTERIC SITES ON MUSCARINIC RECEPTORS

(57) Abstract: An allosteric site on muscarinic receptors is disclosed, together with its use for screening for compounds capable of modulating the binding of a primary ligand such as acetylcholine to the receptor. The site is characterised herein a series of indolocarbazoles represented by formula (1) and a series of related compounds represented by formula (2). These compounds are capable of binding to the allosteric site to modulate the binding of a primary ligand to the receptors, showing positive, negative and neutral cooperativity and selectivity for muscarinic receptor subtypes.

<u>Allosteric Sites on Muscarinic Receptors</u>

<u>Field of the Invention</u>

The present invention relates to muscarinic receptors,
and in particular to compounds which are capable of
binding to an allosteric site on a muscarinic receptor
and modulating the binding of a primary ligand such as
acetylcholine to the receptor. The present invention
further relates to methods for aiding in the
identification of compounds which bind to the allosteric
site and their use in methods of medical treatment.

<u>Background of the Invention</u>

The five muscarinic receptors subtypes are designated M_1-
M_5 and all are activated by the binding of acetylcholine
(ACh). These receptor subtypes are widely distributed in
the central nervous system and in the periphery where
they mediate a number of important physiological
functions. As a consequence these receptors are a
therapeutic target for the treatment of a variety of
conditions and potential therapeutic agents are both
agonists and antagonists. In the treatment of many
conditions it has been thought to be important that the
therapeutic agents have a selective action on one or a
limited number of subtypes. However, there remains a
problem in the art that the muscarinic receptor subtypes
are structurally very similar as a consequence of the
identity of amino acids in the regions of sequence that
are considered to constitute the ACh binding site, i.e.
the site of binding of agonists and competitive
antagonists. Therefore, it has not been possible to
synthesize highly selective muscarinic antagonists and no
directly acting muscarinic agonists of any substantial
selectivity exist (Caulfield and Birdsall, 1998). A
further problem is that synthetic exogenously applied
agonists chronically stimulate receptors and this can

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result in desensitization and downregulation of the
receptor function as well as losing any information
content of the pulsatile endogenous ACh signalling
mechanism.

However, in addition to the primary site on these
receptors at which agonists and competitive antagonists
bind, muscarinic receptors are known to also contain an
allosteric site. Compounds binding at the allosteric
site mediate the binding of the ligands to the primary
binding site (GB 2 292 685 A and WO 96/03377). Thus, it
is possible that compounds binding at the allosteric site
may overcome some of these problems involved in selective
modulation of muscarinic receptor subtypes. By way of
example, it is known that when brucine and some of its N-
substituted analogues bind at the allosteric site, they
modulate the response of muscarinic receptors to the
primary ligand acetylcholine (ACh) or N-methylscopolamine
(NMS), a competitive antagonist of ACh. The modulation
caused by compounds binding at the allosteric site can be
positive, negative or neutral. A compound which has
neutral cooperativity with ACh at one muscarinic receptor
subtype binds to the receptor but has no action at any
concentration. In contrast, if the same ligand has
positive or negative cooperativity at another subtype it
has an action at that subtype which is totally selective.
This form of selectivity based on cooperativity can be
termed 'absolute subtype selectivity'.. Thus, the
allosteric agents can modulate the interaction between
the muscarinic receptor and the primary ligand.

Summary of the Invention

Broadly, the present invention relates to the finding
that muscarinic receptors have a further allosteric site
which is characterised herein using compound 1a, and a

series of related indolocarbazoles represented by formula
1, and compounds 2a and 2b, and a series of related
compounds represented by formula 2. These compounds are
capable of binding to the allosteric site to modulate the

5   binding of a primary ligand to the receptors, showing
positive, negative and neutral cooperativity and
selectivity for muscarinic receptor subtypes.

Compounds represented by formula 1:

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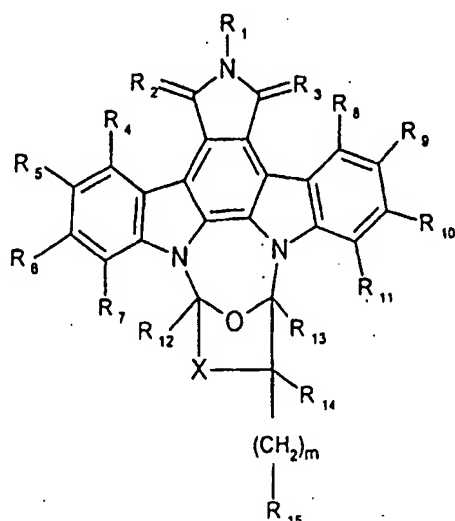
wherein:

R_1 is hydrogen, lower alkyl, aralkyl, iminoalkyl or

25   an imino protecting group such as an acyl group $R_{19}CO$,
where R_{19} is alkyl or aralkyl;

R_2 and R_3 are independently an oxygen or two hydrogen
atoms;

R_4-R_{11} are independently selected from hydrogen or

30   general aromatic substituents e.g. halo, nitro, cyano,
lower alkyl, haloalkyl, alkoxy, hydroxy, aralkoxy;

R_{12} is H or lower alkyl;

X is CH_2,

$CH(OR_{16})$, where R_{16} is hydrogen, lower alkyl or

35   an O-protecting group,
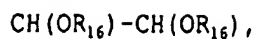
$CH(OR_{16})-CH(OR_{16})$,

$CH(OR_{16})-CH(CH_2)_n-N(R_{17})_2$, where n is an integer between 0-5 and R_{17} is one or two of the following; alkyl, aralkyl;

5   R_{14} is hydrogen, OR_{16}, NHR_{17}, where R_{17} is alkyl, haloalkyl, aralkyl or an acyl group;

m is an integer between 0-8; and,

R_{15} is hydrogen, CO_2R_{18}, $CONHR_{18}$ or an isostere for an ester or substituted ester, where R_{18} is hydrogen, alkyl

10   or substituted alkyl, aralkyl or substituted aralkyl.

Compounds represented by formula 2:

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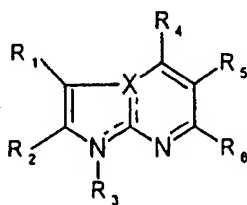
20

wherein:

X is N(H) or C(H);

R_1, R_2 are independently hydrogen, general aromatic substituents e.g. halo, nitro, cyano, lower alkyl,

25   haloalkyl, alkoxy, hydroxy, aralkoxy;

R_3 is hydrogen, alkyl, iminoalkyl, or aralkyl;

R_1 and R_2 together and/or R_2 and R_3 together are a fused aromatic or heterocyclic system optionally with ring substituents;

30   R_4, R_5, R_6 are normal aromatic substituents;

or R_5, R_6 together are a fused alicyclic system with 1-5 rings including steroid ring systems, preferably with substituents at either or both 17α and 17β positions, e.g. hydroxy, alkoxy, aralkoxy, alkyl, alkenyl, or

35   alkynyl, and R_4 is a substituent compatible with the

synthetic method;

or R_5, R_6 together are a fused alicyclic system with 1-5 rings and R_4 is hydrogen or an alkyl group;

or R_4, R_5, R_6 are part of a fused alicyclic system;

and a dotted line indicated either the presence or the absence of a bond.

Preferably, the above generally defined substituents are C_1-C_{10}, or in the case of lower alkyl substituents, C_1-C_6; and in either case, optionally including branching and/or halogen substitution.

The formulae of compounds 1a, 2a and 2b are shown on pages 54 to 56.

Accordingly, in a first aspect, the present invention provides a compound represented by formula 1 or 2 for use in a method of medical treatment.

In a further aspect, the present invention provides the use of a compound for the preparation of a medicament for the treatment of a condition mediated by the binding of a primary ligand to a muscarinic receptor, wherein the compound binds to an allosteric site of the muscarinic receptor which is capable of binding to compound 1a and/or 2a and thereby modulates the binding of the primary ligand to the muscarinic receptor. Preferred compounds include those represented by formula 1 or 2.

In a further aspect, the present invention provides a method of modulating the response of a muscarinic receptor to a primary ligand, the method comprising contacting the muscarinic receptor with a compound which binds to an allosteric site of the muscarinic receptor which is capable of binding to compound 1a and/or 2a and

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which thereby modulates the binding of the primary ligand
to the muscarinic receptor. This method·may be carried
out *in vitro*, e.g. as part of a screening method or to
otherwise activate or modulate the response of the
5   receptor, or *in vivo*, e.g. in the treatment of a patient
suffering from one of the conditions described herein.

The primary ligand binding to the muscarinic receptor may
be an agonist or an antagonist of the receptor's
10 biological activity. Examples of primary ligands include
acetylcholine (ACh) or N-methylscopolamine (NMS). Other
primary ligands are well known to those skilled in the
art.

15 The use of a compound which binds to this allosteric
site, and in particular an allosterically acting compound
which has a positive or negative cooperative effect on
the binding of the primary ligand, can have the advantage
of selectively modulating the natural function of a
20 limited group of the muscarinic receptor subtypes, and
more preferably only a single muscarinic receptor
subtype. Thus, the invention helps to solve the problem
of selectively activating the function of specific
muscarinic receptor subtypes in a way which is difficult
25 to achieve using a primary ligand which binds to multiple
receptor subtypes, and opens up the possibility of
therapeutic treatment based on this selectivity.
Examples of these conditions are discussed below. While
it is generally preferred that the binding of the
30 allosteric compound to the muscarinic receptor enhances
the binding of the primary ligand (i.e. shows positive
cooperativity), compounds which decrease the binding of
the primary ligand (i.e. act antagonistically or show
negative cooperativity) can also have therapeutic
35 potential. Such compounds have the property of not

blocking 100% of the receptor response when they bind to
the allosteric site of the receptor (as competitive
antagonists do). These compounds can be used in the
treatment of conditions including Alzheimer's disease,
motion sickness, depression, bronchitis, gastric and
duodenal ulcers, non ulcer dyspepsia, urinary bladder
incontinence and retention, sinus bradycardia,
Parkinson's disease, incontinence, asthma, chronic
obstructive pulmonary disease, irritable bowel syndrome,
excessive vagal drive, as a preanaesthetic, for cardiac
pacemaker regulation, or for the regulation of sleep.

The allosteric site defined herein is distinct from the
'common allosteric site' disclosed in the prior art (e.g.
GB 2 292 685 A) which binds to gallamine, strychnine,
brucine and N-substituted brucine analogues. In
contrast, the present inventors have found a new
allosteric site which binds compound 1a and a series of
related indolocarbazoles having formula 1 and compound 2a
and a series of related compounds having formula 2.

Preferably, the muscarinic receptor is selected from the
M_1, M_2, M_3, M_4 or M_5 receptors known in the art. The
receptors may be human or an appropriate animal homologue
(rat, mouse etc). The generation of transfected cell
lines stably or transiently expressing one or more of the
M_1-M_5 receptor genes from any given species is well in the
art and relevant references are cited, for example, in
the reviews: Hulme et al, 1990 and Caulfield and
Birdsall, 1998.

Exemplary compounds which are capable of modulating the
binding of a primary ligand to a muscarinic receptor by
interaction with the allosteric site described for the
first time herein include compounds 1a, 2a and 2b.

In a further aspect, the present invention provides a
method for aiding in the identification of compounds
capable of modulating the binding of a primary ligand to
a muscarinic receptor by binding to an allosteric site of

5   the muscarinic receptor which is capable of binding to
compound 1a and/or compound 2a, the method comprising:

(a)   contacting the muscarinic receptor and the
primary ligand with one or more concentrations of a
candidate compound; and,

10   (b)   determining whether the candidate compound
modulates the binding of a primary ligand to the
muscarinic receptor by binding to the allosteric site of
the receptor which is capable of binding compound 1a
and/or compound 2a.

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The method may comprise the further step of selecting a
candidate compound which modulates the binding of the
primary ligand to the muscarinic receptor.

20   In a further aspect, the present invention provides the
use of an allosteric site of a muscarinic receptor which
is capable of binding to compound 1a and/or 2a in
screening for compounds which are capable of modulating
the binding of a primary ligand to a muscarinic receptor

25   by binding to the allosteric site.

The modulation of the binding of the primary ligand may
be achieved by a number of mechanisms. The allosteric
compound may have a positive or negative cooperative

30   effect at one or more of the muscarinic receptor
subtypes, and preferably no effect (neutral
cooperativity) at other receptor subtypes. Alternatively
or additionally, the binding of the allosteric compound
may affect the binding of an agent acting at a different

35   allosteric site such as the common allosteric site which

binds brucine. .

Preferably, candidate allosteric compounds are selected
if they have a positive or negative cooperative effect on
5   the binding of the primary ligand. Thus, in preferred
embodiments, step (b) involves determining whether the
candidate compounds bind to the allosteric site and
additionally determining how the binding modulates the
action of the primary ligand at its binding site. This
10   can be carried out using the assays described herein
including equilibrium and/or kinetic binding assays
and/or functional assays.

General assays which are suitable or can be adapted for
15   use in the present invention are described in Lazareno
and Birdsall (1995), Detection, quantitation and
verification of allosteric interactions of agents with
labelled and unlabelled ligands at G-protein-coupled
receptors: Interactions of strychnine and acetylcholine
20   at muscarinic receptors. Mol. Pharmacol. 48:362-378;
Lazareno et al (1998), Subtype selective positive
cooperative interactions between brucine analogues and
acetylcholine at muscarinic receptors: radioligand
binding studies. Mol. Pharmol. 53:573-589; Birdsall et
25   al (1999), Subtype selective positive cooperative
interactions between brucine analogues and acetylcholine
at muscarinic receptors: functional studies. Mol.
Pharmacol. 55:778-786.

30   Examples of specific assays which can be employed are
described in the 'Materials and Methods' section below.

Firstly, the candidate compounds are selected as being
allosteric using the criteria defined in the above
35   general and specific assays. The compounds are further

selected as binding to the novel site, e.g. the site
which binds compound 2a, using specific kinetic and
equilibrium assays. For example, any allosteric compound
which is not competitive with the binding of brucine,

5   gallamine or strychnine and is competitive with the
binding of 2a to the novel allosteric site is a candidate
compound for further investigation or development as a
therapeutic. These methods are useful for finding
compounds which are positively, negatively or neutrally

10   cooperative with the binding of the primary ligand.

In preferred embodiments, the screening is carried out
using a receptor, one or more concentrations of a
candidate allosteric ligand (possibly including an assay

15   carried out in the absence of the candidate ligand by way
of control) and one or more primary ligands, in the
presence or absence of another allosteric ligand.
Preferably, the primary ligand employed in this screening
method is acetylcholine (ACh) and/or N-methylscopolamine

20   (NMS), although other suitable primary ligands are well
known to those skilled in the art. In one embodiment,
the method involves as assay in which the binding of a
candidate compound to the allosteric site is determined
in using labelled NMS in the absence or presence of one

25   or more concentrations of ACh. Alternatively or
additionally, the binding of a candidate compound to the
allosteric site is determined using labelled NMS in
assays which determine the NMS dissociation rate constant
in the presence and absence of one or more concentrations

30   of the candidate compound. Another preferred assay
format is an assay of the effects of one or more
concentrations of an allosteric ligand on the
acceleration of the dissociation rate of NMS from
muscarinic receptors produced by 2a (described in Part II

35   below). A further assay is to quantitate the effects of

a test compound, which has been demonstrated in the
general assays to be allosteric, on the equilibrium
allosteric effects of ligands which are known to bind one
or other of the two allosteric sites described in Part I,

5   Figure 5).

In these methods, the candidate compound may be selected
if it enhances the binding of the primary ligand to the
muscarinic receptor (otherwise referred to as positive

10   cooperativity). However, other compounds may be selected
if they reduce the binding of the primary ligand to the
muscarinic receptor (otherwise referred to as negative
cooperativity). Candidate compounds having neutral
cooperativity are selected if they bind to one or more of

15   the muscarinic receptor subtypes but have no action on
the equilibrium binding of a primary ligand at any
concentration.

The allosteric site employed in the work described herein

20   is capable of binding compounds 1a and/or 2a. In
contrast to the first allosteric site disclosed in the
prior art, the site described herein does not bind to
brucine, gallamine and/or strychnine to any substantial
extent at concentrations up to $10^{-4}M$.

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In a further aspect, the present invention provides a
method which comprises, having identified a candidate
compound by the above method, the further step of
manufacturing the compound in bulk and/or formulating the

30   compound as a pharmaceutical composition.

In a further aspect, the present invention provides the
use of a compound as obtainable by the above method for
the preparation of a medicament for the treatment of a

35   conditions mediated by the binding of the primary ligand

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to the muscarinic receptor. Examples of these conditions discussed below.

Embodiments of the invention will now be described by way of example and not limitation with reference to the accompanying drawings.

Brief Description of the Figures

Figure 1: Effect of staurosporine (1f) on the binding of ^{3}H-NMS (210 pM) at M_1 receptors in the absence and presence of 2.2 mM ACh, all in the presence of 0.2 mM GTP. The points are individual observations. The lines show the fit to Equation 2 (see Methods), which yielded a log affinity of 5.95 ± 0.06, a slope factor of 1.01 ± 0.05, cooperativity with ^{3}H-NMS of 1.51 ± 0.06, and cooperativity with ACh of 0.27 ± 0.03. The affinity ratio plots of these data are shown in Figure 2.

Figure 2: Affinity ratio plots of five indolocarbazoles (1a, 1b, 1e, 1f and 1i) at M_1-M_4 receptors. The points were derived from duplicate observations of ^{3}H-NMS binding in the absence and presence of ACh, as described in Methods. The parameter estimates pK (log affinity of the test agent for the free receptor), α_{NMS} (cooperativity with ^{3}H-NMS), and β_{ACh} (cooperativity with ACh) were derived from nonlinear regression analysis with Equations 1 or 2 as appropriate (see Methods). The parameter estimates from a number of similar assays are summarised in Table 1.

Figure 3: Effect of 1a, 1b, 1e, 1f and 1i on the dissociation rate constant (k_{off}) of ^{3}H-NMS at M_1-M_4 receptors, expressed as a percent inhibition of the control k_{off}. The points are the mean and range/2 of duplicate observations. The lines show the fit to a

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logistic function, as described in Methods. The parameter
estimates from a number of similar assays are summarised
in Table 2.

5   Figure 4: Effect of various concentrations of KT5720 (1a)
     on the inhibition of ^{3}H-NMS (50 pM) binding at M_1
     receptors by ACh in a volume of 3 ml. The points are the
     mean and range/2 of duplicate observations. The lines
     show the fit to Equation 1 with the slope factor for
10   KT5720 binding set to 1. The parameter estimates were:
     log affinity of KT5720 6.6 ± 0.1, cooperativity with
     ^{3}H-NMS 1.9 ± 0.1, cooperativity with ACh 1.6 ± 0.2. The
     inset shows affinity ratio plots derived from these
     parameters (see Methods). The -log IC_{50} values of ACh in
15   the presence of increasing concentrations of KT5720, from
     independent logistic fits of the curves, were 5.28, 5.33,
     5.40 and 5.42.

     Figure 5: Inhibition by gallamine of ^{3}H-NMS binding at M_1
20   receptors in the presence of various concentrations of
     (A) KT5720 (1a) and (B) staurosporine (1f). The points
     are individual observations. The lines show the fit of
     the data to Equation 3, where the cooperativity estimates
     of gallamine with KT5720 and staurosporine were not
25   significantly different from 1 and 0 respectively and
     were set to those values. The slope factors for
     gallamine, KT5720 and staurosporine were not different
     from 1 and were set at that value. From three such
     assays, similarly constrained, KT5720 had a log affinity
30   of 6.22 ± 0.17 and cooperativity with ^{3}H-NMS of 2.39 ±
     0.08. From three such assays, similarly constrained,
     staurosporine had a log affinity of 5.75 ± 0.11 and
     cooperativity with ^{3}H-NMS of 1.62 ± 0.13. From these six
     assays gallamine had a log affinity of 5.05 ± 0.05 and
35   cooperativity with ^{3}H-NMS of 0.11 ± 0.01. The insets show

the effect of the test agent on the $-\log IC_{50}$ of
gallamine, obtained from nonlinear regression analysis of
the individual curves.

5   Figure 6: Effect of KT5720 (1a) on ^3H-NMS dissociation
from M_1 receptors, alone and in the presence of other
allosteric agents, measured at a single time point as
described in Methods. The points show the mean and s.e.m
of quadruplicate observations obtained in two assays,
10   except for $10^{-4}M$ gallamine, and $3.10^{-5}M$ and $3.10^{-4}M$ brucine
which show the mean and range/2 of duplicate
observations. The lines in the top panel show the fits of
the individual curves to a hyperbolic function, except
for those in the presence of staurosporine. The estimates
15   of the log EC_{50} of KT5720 derive from those fits. The top
panel shows the data as % inhibition of the control k_{off}
of ^3H-NMS. The lower panel shows shows Ef, the ^3H-NMS k_{off}
values in the presence of KT5720 and a certain
concentration of test agent (gallamine, brucine or
20   staurosporine) as a fraction of the k_{off} values in the
presence of that concentration of test agent alone.

Figure 7: Concentration-dependent effects of the
compounds shown on pages 55 and 56 on the ^3H-NMS-occupied
25   receptor. The percent inhibition of the dissociation
rate constant of ^3H-NMS from M_1-M_4 receptors was measured
at a single time point. The legends indicate the IC_{50} (or
EC_{50}) values obtained using nonlinear regression analysis
of these curves.

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Figures 8 and 9: Concentration-dependent effects of
active compounds on the equilibrium binding of ^3H-NMS and
ACh. The effects are expressed as 'affinity ratios', i.e.
the apparent affinity of the 'primary' ligand (^3H-NMS or
35   ACh) in the presence of a particular concentration of

test agent divided by its apparent affinity in the
absence of test agent. Affinity ratios were calculated
as described in 'Methods'. Affinity ratios > 1 indicate
positive cooperativity, affinity ratios < 1 indicate
5   negative cooperativity, and affinity ratios of 1 with one
primary ligand at concentrations of test agent which
modify the binding of the other primary ligand indicate
neutral cooperativity. The IC_{50}, or EC_{50}, of a test agent
on the affinity ratio of either primary ligand
10   corresponds approximately to the Kd of the test agent for
the free receptor. High concentrations of compounds
which show neutral or positive cooperativity with ^3H-NMS
and which strongly inhibit ^3H-NMS dissociation may inhibit
^3H-NMS binding through a kinetic effect, i.e. lack of
15   equilibration of ^3H-NMS binding.

Figure 10: Inhibition of ^3H-NMS binding to M_3 receptors by
ACh, alone and in the presence of three concentrations of
2c (WIN 62577). GTP (0.2 mM) was present. The data were
20   fitted to Equation 1 (see Methods) to yield a log
affinity of 2c of 5.31, cooperativity with ^3H-NMS of 0.47,
and cooperativity with ACh of 1.41. The inset shows
affinity ratios (1/dose ratio) for ACh and ^3H-NMS,
calculated from the parameters of the fit.
25

Figure 11: Dissociation of ^3H-NMS over time, alone and in
the presence of three concentrations of 2a. For each
receptor subtype, the parameter estimates and standard
errors were derived from the nonlinear regression fits of
30   the entire dataset to a version of Equations 2 and 3,
where k_{off} and k_{offx} are the dissociation rate constants of
^3H-NMS from the free and 2a-liganded receptor
respectively, and pK_{occ} is the log Kd of 2a for the ^3H-NMS-
occupied receptor. The insets show the linearising
35   transformation ln(B/Bo) vs. time, where B is the specific

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binding remaining after a certain time and Bo is the
initial level of specific binding.

Figure 12: Concentration-effect curves for 2a on ^{3}H-NMS
dissociation, alone and in the presence of one or three
concentrations of test agent, measured at a single time
point. The data were converted to dissociation rate
constants (see Methods) and expressed as a % of the
control dissociation rate constant. The curves show the
fits from nonlinear regression analysis to Equation 1.
For strychnine and gallamine, dissociation of ^{3}H-NMS from
the dually or triply liganded receptor was not different
from 0 and the cooperative interaction with 2a was not
different from 1 (i.e. neutral cooperativity), so these
values were fixed. For 1a (KT 5720), 1f (staurosporine)
and 2b (WIN 51708), the cooperative interaction with 2a
was not different from 0 (i.e. competition), so this
value was fixed. The panel marked 'dose-ratio plots'
shows the log of the ratio $EC_{50, +agent}/EC_{50\_alone}$ of 2a vs
log[agent]. Each assay was repeated at least once, with
similar results.

Detailed Description
Assays

As indicated above, the screening assays of the invention
are useful in the identification of compounds capable of
binding to the allosteric site disclosed herein and
modulating the binding of a primary ligand to a
muscarinic receptor. The precise format of these assays
can be readily devised by the skilled person using the
common general knowledge in the art. In one embodiment,
the assays will employ (a) a muscarinic receptor of one
of the M_1 to M_5 subtypes, (b) one or more primary ligands
or ligand analogues, and (c) one or more candidate
compounds. Optionally, the assays may additionally

employ (d) a compound known to act at the allosteric site
which is capable of competing with the candidate compound
being tested. The assays will generally involve
contacting the receptor, the primary ligands or ligand
5   analogues and one or more concentrations of the candidate
compound *in vitro*, under conditions in which the
candidate compound can bind or compete for binding at the
allosteric site. The results of the assays can be
determined by labelling one or more of the candidate
10   compound, the competitive allosteric compound or the
primary ligand or ligand analogue, and determining which
species interact in the assay system.

In an alternative embodiment, and especially in the
15   context of high throughput screening, it may be desirable
that the screening assays involve determining whether the
candidate compound binds to the allosteric site disclosed
herein in the absence of the primary ligand. These
assays could be followed with a separate determination of
20   whether or in what sense the compounds binding to the
allosteric site modulate the binding of a primary ligand
or ligand analogue to the receptor. These assays could
be carried out by contacting (a) a muscarinic receptor
and (b) one or more candidate compounds, and optionally,
25   (c) one or more compounds known to act allosterically,
under conditions in which compounds (b) and/or (c) can
bind or compete for binding to the allosteric site. The
binding of the candidate compound or competitive compound
to the receptor can be determined by labelling compounds
30   (b) and/or (c).

The above assays may comprise carrying out controls, e.g.
carrying out the assay in the absence or presence of the
candidate compound(s).

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In these assays, the muscarinic receptors can be present
in either a free form or alternatively immobilised, e.g.
on the surface of a cell expressing the receptor, or a
solid support. A preferred format uses cell surface
5   receptors.

The labelling of different types of agents is well known
in the art. Broadly, this involves tagging the agent
with a label or reporter molecule which can directly or
10   indirectly generate detectable, and preferably
measurable, signal. The linkage of reporter molecules
may be direct or indirect, e.g. by a covalent bond or a
non-covalent interaction. Examples of commonly used
labels include fluorochrome, phosphor or laser dyes with
15   spectrally isolated absorption or emission
characteristics. Suitable fluorochromes include
fluorescein, rhodamine, luciferin, phycoerythrin and
Texas Red. Suitable chromogenic dyes include
diaminobenzidine. Other detectable labels include
20   radioactive isotopic labels, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{126}I,
or ^{99m}Tc, and enzyme labels such as alkaline phosphatase,
β-galactosidase or horseradish peroxidase, which catalyze
reactions leading to detectable reaction products and can
provide amplification of signal.
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Other reporters include macromolecular colloidal
particles or particulate material such as latex beads
that are coloured, magnetic or paramagnetic, and
biologically or chemically active agents that can
30   directly or indirectly cause detectable signals to be
visually observed, electronically detected or otherwise
recorded. These molecules may be enzymes which catalyze
reactions that develop or change colour or cause changes
in electrical properties. They may be molecularly
35   excitable, such that electronic transitions between

energy states result in characteristic spectral
absorptions or emissions.

In the context of high throughput screening, the methods
5   described herein can involve carrying out assays using
groups or pools of candidate compounds, rather than
individual compounds, to enhance the rate at which
candidate compounds can be discarded. Individual groups
of compounds having positive results in an assay can then
10   be separated and screened to identify the compound(s) in
the group which interact with the allosteric site and
modulate the binding of the primary ligand. Appropriate
measures should be taken to ensure that any one candidate
compound is assayed with different pools of other
15   candidate compounds. This protocol minimises the
possible interfering masking effects of competitive
antagonists or agonists which may be in one pool but not
another.

20   The candidate compounds used may be natural or synthetic
chemical compounds used in drug screening programmes.
Mixtures of naturally occurring materials which contain
several characterised or uncharacterised components may
also be used.
25

Other candidate compounds may be based on modelling the
3-dimensional structure of a polypeptide or peptide
fragment and using rational drug design to provide
candidate compounds with particular molecular shape,
30   size, hydrophobicity, hydrophilicity and charge
characteristics.

The amount of candidate compound which may be added to an
assay of the invention will normally be determined by
35   trial and error depending upon the type of compound used.

Typically, from about 0.01 to 100,000nM concentrations of candidate compound may be used, for example 0.1 to 100μM.

In a further step, the method of the present invention
5   may involve quantifying the amount of a candidate compound required to modulate the binding of the primary ligand to the muscarinic receptor by more detailed equilibrium or kinetic assays.

10   The screening methods of the invention may be followed by isolation and/or manufacture and/or use of a candidate compound selected in an assay, and/or further testing to determine whether a candidate compound having a positive, neutral or negative cooperative effect on the binding of
15   the primary ligand to the muscarinic receptor has a biological property which makes it suitable for further development as a lead compound. These include tests to determine its activity in assays of function in membranes, whole cells, whole tissues and/or in vivo, as
20   well as tests of its metabolic stability, bioavailability and duration of action and for the presence of side effects.

In a further aspect, the present invention provides the
25   use of the above compounds in the design or screening for mimetics of the compounds which share the property of binding to the allosteric site of muscarinic receptors which binds to compound 1a and/or compound 2b.

30   The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a lead compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a
35   particular method of administration. Mimetic design,

synthesis and testing may be used to avoid randomly
screening large number of molecules for a target
property.

5   There are several steps commonly taken in the design of a
mimetic from a compound having a given target property.
Firstly, the particular parts of the compound that are
critical and/or important in determining the target
property are determined. These parts or residues
10   constituting the active region of the compound are known
as its pharmacophore. Once the pharmacophore has been
found, its structure is modelled to according its
physical properties, e.g. stereochemistry, bonding, size
and/or charge, using data from a range of sources, e.g.
15   spectroscopic techniques, X-ray diffraction data and NMR.
Computational analysis, similarity mapping (which models
the charge and/or volume of a pharmacophore, rather than
the bonding between atoms) and other techniques can be
used in this modelling process.
20

In a variant of this approach, the three-dimensional
structure of the ligand and its binding partner are
determined or modelled. This can be especially useful
where the ligand and/or binding partner change
25   conformation on binding, allowing the model to take
account of this the design of the mimetic.

A template molecule is then selected onto which chemical
groups which mimic the pharmacophore can be grafted. The
30   template molecule and the chemical groups grafted on to
it can conveniently be selected so that the mimetic is
easy to synthesise, is likely to be pharmacologically
acceptable, and does not degrade *in vivo*, while retaining
the biological activity of the lead compound. The
35   mimetic or mimetics found by this approach can then be

screened to see whether they have the target property, or
to what extent they exhibit it. Further optimisation or
modification can then be carried out to arrive at one or
more final mimetics for further testing or optimisation,
e.g. *in vivo* or clinical testing.

Pharmaceutical Uses

The compounds identified herein as being useful for
modulating the binding of a primary ligand to a
muscarinic receptor can be formulated and used as
pharmaceuticals.

The pharmaceutical compositions may comprise, in addition
to one or more of the compounds, a pharmaceutically
acceptable excipient, carrier, buffer, stabiliser or
other materials well known to those skilled in the art.
Such materials should be non-toxic and should not
interfere with the efficacy of the active ingredient.
The precise nature of the carrier or other material may
depend on the route of administration, e.g. oral,
intravenous, cutaneous or subcutaneous, nasal,
intramuscular, or intraperitoneal routes.

Pharmaceutical compositions for oral administration may
be in tablet, capsule, powder or liquid form. A tablet
may include a solid carrier such as gelatin or an
adjuvant. Liquid pharmaceutical compositions generally
include a liquid carrier such as water, petroleum, animal
or vegetable oils, mineral oil or synthetic oil.
Physiological saline solution, dextrose or other
saccharide solution or glycols such as ethylene glycol,
propylene glycol or polyethylene glycol may be included.

The pharmaceutical formulations can be prepared by mixing
the compounds of the present invention with one or more

adjuvants, such as excipients (e.g. organic excipients
including sugar derivatives, such as lactose, sucrose,
glucose, mannitol or sorbitol; starch derivatives, such
as corn starch, dextrine or carboxymethyl starch;

5   cellulose derivatives, such as crystalline cellulose, low
hydroxypropyl-substituted cellulose, carboxymethyl
cellulose, carboxymethyl cellulose calcium or internally
bridged carboxymethyl cellulose sodium; gum arabic;
dextran; and Pullulan; inorganic excipients including

10   silicates, such as light silicic acid anhydride,
synthetic aluminium silicate or magnesium meta-silicic
acid aluminate; phosphates, such as calcium phosphate;
carbonates, such as calcium carbonate; and sulphates,
such as calcium sulphate); lubricants (e.g. metal

15   stearates, such as stearic acid, calcium stearate or
magnesium stearate; talc; colloidal silica; waxes, such
as beeswax or spermaceti; boric acid; adipic acid;
sulphates, such as sodium sulphate; glycol; fumaric acid;
sodium benzoate; DL-leucine; sodium salts of aliphatic

20   acids; lauryl sulphates, such as sodium laurylsulphate or
magnesium laurylsulphate; silicates, such as silicic acid
anhydride or silicic acid hydrate; and the foregoing
starch derivatives); binders (e.g. polyvinyl pyrrolidone,
Macrogol; and similar compounds to the excipients

25   described above); disintegrating agents (e.g. similar
compounds to the excipients described above; and
chemically modified starch-celluloses, such as
Crosscarmelose sodium, sodium carboxymethyl starch or
bridged polyvinyl pyrrolidone); stabilisers (e.g. p-

30   hydroxybenzoates, such as methylparaben or propylparaben;
alcohols, such as chlorobutanol, benzyl alcohol or
phenylethyl alcohol; benzalkonium chloride; phenols, such
as phenol or cresol; thimerosal; dehydroacetic acid; and
sorbic acid); corrigents (e.g. sweeteners, vinegar or

35   perfumes, such as conventionally used); diluents and the

like.

For intravenous, cutaneous or subcutaneous injection, or
injection at the site of affliction, the active
5   ingredient will be in the form of a parenterally
acceptable aqueous solution which is pyrogen-free and has
suitable pH, isotonicity and stability. Those of
relevant skill in the art are well able to prepare
suitable solutions using, for example, isotonic vehicles
10   such as sodium chloride injection, Ringer's injection,
lactated Ringer's injection. Preservatives, stabilisers,
buffers, antioxidants and/or other additives may be
included, as required.

15   Preferably, the pharmaceutically useful compound
according to the present invention is given to an
individual in a 'prophylactically effective amount' or a
'therapeutically effective amount' (as the case may be,
although prophylaxis may be considered therapy), this
20   being sufficient to show benefit to the individual.
Typically, this will be to cause a therapeutically useful
effect in the patient, e.g. using the compounds to
regulate the action of the primary ligand at a muscarinic
receptor, and preferably one of the muscarinic receptor
25   subtypes. The actual amount of the compounds
administered, and rate and time-course of administration,
will depend on the nature and severity of the condition
being treated. Prescription of treatment, e.g. decisions
on dosage etc, is within the responsibility of general
30   practitioners and other medical doctors, and typically
takes account of the disorder to be treated, the
condition of the individual patient, the site of
delivery, the method of administration and other factors
known to practitioners. Examples of the techniques and
35   protocols mentioned above can be found in Remington's

Pharmaceutical Sciences, 16th edition, Oslo, A. (ed),
1980.

In particular, the compounds may be useful in the
5   treatment of conditions mediated by the action of ACh at
a muscarinic receptor. By way of example, these include
Alzheimer's disease, Parkinson's disease, motion.
sickness, Huntingdon's chorea, schizophrenia, depression,
anxiety, sedation, analgesia, stroke, preanaesthetic,
10   antispasmodic, irritable bowel syndrome, bladder-
incontinence or retention, peptic ulcer disease,
bronchitis/asthma/chronic obstructive airway disease,
sinus bradycardia, pacemaker regulation, glaucoma,
achalasia, symptomatic diffuse oesophageal spasm, biliary
15   dyskinesia, scleroderma, diabetes mellitus, lower
oesophageal incompetence, intestinal pseudo obstruction,
regulation of sleep, control of pupil diameter and non-
ulcer dyspepsia.

20   Depending on the type and severity of condition, the
composition can be administered to provide an initial
dose of about 0.01 to 20 mg, more preferably 0.02 to 10
mg, of compound/kg of patient weight. As mentioned
above, other dosing regimens and the determination of
25   appropriate amount of the compounds for inclusion in the
compositions can be readily determined by those skilled
in the art.

Materials
30   ^{3}H-NMS (81-86 Ci/mmol) was from Amersham International,
UK, and ^{35}S-GTPγS (1000-1400 Ci/mmol) was from NEN,
Boston. Brucine sulfate, gallamine triiodide and ACh
chloride were from Sigma Chemical Co., Dorset, UK.
Staurosporine was from Sigma and from Alexis Corporation,
35   Nottingham, UK. Gö 7874 (1i), Gö 6976 (1h) and K-252c

(1g) were from Calbiochem, Nottingham, UK.   K-252a (1b)
and K-252b (1c) were from Alexis and from TCS Biologicals
Ltd, Buckingham, UK.   KT5823 (1e) and KT5720 (1a) were
from TCS, Calbiochem and Alexis.   KT5926 (1d) was from

5   TCS and Calbiochem.   WIN 51708 (2b) and WIN 62577 (2c)
were from RBI (Semat), St Albans, UK.   Analogues of 2
were synthesised using literature methods according to
the general scheme:

10

15
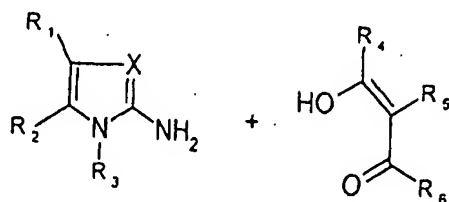
20   with further chemical modification of the product as
necessary.   Further assistance for the synthesis of the
compounds or related ones is provided in the papers by
Bajwa and Sykes (1978-1980) cited in the references
section.

25

Methods

Cell culture and membrane preparation:
CHO cells stably expressing cDNA encoding human
muscarinic M_1-M_4 receptors (Buckley et al, 1989) were
30   grown in alpha-MEM medium (GIBCO) containing 10% (v/v)
new born calf serum, 50 U/ml penicillin, 50 µg/ml
streptomycin and 2 mM glutamine, at 37° under 5% CO_2.
Cells were grown to confluence and harvested by scraping
in a hypotonic medium (20 mM Hepes + 10 mM EDTA, pH 7.4).
35   Membranes were prepared at 0°C by homogenization with a

Polytron followed by centrifugation (40,000 x g, 15 min),
were washed once in 20 mM Hepes + 0.1 mM EDTA, pH 7.4,
and were stored at -70°C in the same buffer at protein
concentrations of 2-5 mg/ml. Protein concentrations were
5   measured with the BioRad reagent using bovine serum
albumin as the standard. The yields of receptor varied
from batch to batch but were approximately 10, 1, 2 and 2
pmol/mg of total membrane protein for the M_1, M_2, M_3 and M_4
subtypes respectively.
10

Radioligand binding assays:
Unless otherwise stated, frozen membranes were thawed,
resuspended in incubation buffer containing 20 mM Hepes +
100 mM NaCl + 10 mM $MgCl_2$ (pH 7.4) and incubated with
15   radioligand and unlabelled drugs for two hours at 30°C in
a volume of 1 ml. Membranes were collected by filtration
over glass fibre filters (Whatman GF/B) presoaked in 0.1%
polyethylenimine, using a Brandel cell harvester (Semat,
Herts, UK), extracted overnight in scintillation fluid
20   (ReadySafe, Beckman) and counted for radioactivity in
Beckman LS6000 scintillation counters. Membrane protein
concentrations (5-50 μg/ml) were adjusted so that not
more than about 15% of added radioligand was bound.
Nonspecific binding was measured in the presence of 10^{-6}M
25   QNB (an antagonist with picomolar potency) and accounted
for 1-5% of total binding. GTP was present at a
concentration of 2×10^{-4}M in assays containing unlabelled
ACh. Data points were usually measured in duplicate.
CHO cell membranes do not possess cholinesterase activity
30   (Gnagey and Ellis, 1996; Lazareno and Birdsall, 1993) so
ACh could be used in the absence of a cholinesterase
inhibitor. The compounds were dissolved in dimethyl
sulfoxide which, at the highest final concentration of
2%, had no effect on binding.
35

Experimental designs and data analysis:

General data preprocessing, as well as the 'affinity
ratio' calculations and routine plots of the
semiquantitative equilibrium assay, were performed using
Minitab (Minitab Ltd, Coventry, UK). The other assays
were analysed with nonlinear regression analysis using
the fitting procedure in SigmaPlot (SPSS Inc., Erkrath,
Germany). This procedure is relatively powerful in that
it allows the use of two or more independent variables,
e.g. concentrations of two drugs.

**Equilibrium binding assays for estimation of the affinity
of an allosteric agent for the receptor and the magnitude
of its cooperativity with ^{3}H-NMS and ACh:**

The design and analyses have been described in detail
(Lazareno and Birdsall, 1995; Lazareno et al, 1998).
Briefly, specific binding of a low concentration of ^{3}H-NMS
(1-2 times the Kd) was measured in the presence of a
number of concentrations of test agent, all in the
absence and presence of one or more concentrations of
ACh. Specific binding of a high concentration of ^{3}H-NMS
(5-10 times Kd) was also measured. Nonlinear regression
analysis was used to fit the data to the equation:

Equation 1

$$B_{LAX} = \frac{Bmax.L.K_L.(1+\alpha.(X.K_x)^s)}{1+(X.K_x)^s+(A.K_A)^n.(1+\beta.(X.K_x)^s)+L.K_L.(1+\alpha.(X.K_x)^s)}$$

where B_{LAX} is observed specific bound radioligand, L, A,
and X are concentrations of ^{3}H-NMS, ACh and allosteric
agent respectively, K_L, K_A and K_X are affinity constants
for the corresponding ligands and the receptor, α and β
are allosteric constants of X with ^{3}H-NMS and ACh

respectively, n is a logistic slope factor to describe
the binding of ACh, and s is a 'Schild slope' factor to
describe the binding of X. According to the allosteric
model s should be 1.

Above a certain concentration, some allosteric agents,
especially those which exhibit neutral or positive
cooperativity with ^{3}H-NMS, may slow the kinetics of ^{3}H-NMS
binding so much that the binding does not reach
equilibrium. In most cases sufficient incubation time
was used to allow ^{3}H-NMS binding in the presence of the
agent to reach equilibrium. In a few cases, however, the
highest concentration of agent would be predicted to slow
^{3}H-NMS kinetics sufficiently to prevent binding
equilibrium from being reached, and in these cases the
data were better fitted to the equation:

Equation 2

$$B_{LAXt} = B_{LAX} + (B_{L_o} - B_{LAX}) \cdot \left(\exp\left(\frac{-t.k_{off}}{1 + \alpha.(X.K_x)^s} + \frac{t.k_{off}L.K_L}{1 + (X.K_x)^s + (A.K_A)^n.(1 + \beta.(X.K_x)^s)} \right) \right)$$

where B_{LAXt} is observed specific binding under
nonequilibrium conditions, B_{LAX} is the predicted
equilibrium binding defined in Equation 1, t is the
incubation time, k_{off} is the dissociation rate constant of
^{3}H-NMS, and B_{L0} is the initial amount of bound
radioligand, set to zero in this case. This equation
assumes that the dissociation of ^{3}H-NMS from the
allosteric agent-occupied receptor is negligible, and
that the binding kinetics of both ACh and the allosteric
agent are fast in comparison with the dissociation rate
of ^{3}H-NMS.

If only a single concentration of ACh was used, the data were visualised with 'affinity ratio' plots, where the affinity ratio is the apparent affinity of the 'primary' ligand (^{3}H-NMS or ACh) in the presence of a particular

5　concentration of test agent divided by the apparent affinity of the primary ligand in the absence of test agent. Theoretically, the EC_{50} or IC_{50} of the affinity ratio plot corresponds to the Kd of the test agent at the free receptor, and the asymptotic level corresponds to

10　the cooperativity constant for the test agent and primary ligand (Lazareno and Birdsall, 1995). Affinity ratios were calculated from the specific binding data as follows (Lazareno and Birdsall, 1999):

15　The affinity ratio of ^{3}H-NMS in the presence of a single concentration of test agent is given by the equation:

Equation 3

20
$$r_L = \frac{B_{Lx}(B_{Ll} - B_L)}{B_{Ll} \cdot B_L \cdot (1 - q) - B_{Lx}(B_L - q \cdot B_{Ll})}$$

The affinity ratio of ACh the presence of a single concentration of test agent is given by the equation:

25

Equation 4

$$r_A = \frac{B_L \cdot B_{LA} \cdot (B_{Ll} - B_L) \cdot (B_{Lx} - B_{LAx})}{B_{LAx}(B_L - B_{LA}) \cdot [B_{Ll} \cdot B_L \cdot (1 - q) - B_{Lx}(B_{Lx}(B_L - q \cdot B_{Ll})]}$$

30

where B_L is binding in the presence of the low [^{3}H-NMS] alone; B_{Ll} is binding in the presence of the high [^{3}H-NMS]; B_{LA} is binding in the presence of the low [^{3}H-NMS] and ACh; B_{Lx} is binding in the presence of the low [^{3}H-

35　NMS] and a particular concentration of test agent; B_{LAx} is

binding in the presence of the low [^{3}H-NMS], ACh and the same concentration of test agent; L is the low ^{3}H-NMS concentration; L_1 is the high ^{3}H-NMS concentration; and q is the ratio of low and high ^{3}H-NMS concentrations, L/L_1.

With assays containing a number of ACh concentrations, affinity ratio plots were calculated using the parameter estimates from the fit of the dataset to Equation 1 or 2 as appropriate (Lazareno and Birdsall, 1995). The affinity ratio of ^{3}H-NMS and ACh, r_L and r_A respectively, are given by the equations:

Equation 5

$$r_L = \frac{1 + \alpha.X.K_x}{1 + X.K_x}$$

and equation 6

$$r_A = \frac{1 + \beta.X.K_x}{1 + X.K_x}$$

where the symbols are as described above.

Off-rate assay to estimate the affinity of an allosteric agent for the ^{3}H-NMS-occupied receptor:

A high concentration of membranes (2-4 mg protein/ml) was incubated with a high concentration of ^{3}H-NMS (5 nM) for about 15 minutes. Then 10 µl aliquots were distributed to tubes which were empty or contained 1 ml of 10^{-6}M QNB alone and in the presence of a number of concentrations of allosteric agent (typically n=4). Non-specific binding was measured in separately prepared tubes containing 10 µl membrane and 2 µl of ^{3}H-NMS + QNB. Some time later, about 2.5 dissociation half-lives (see Table 2), the samples were filtered. The data were transformed

32

to rate constants, k_{off}, using the formula:

$$k_{off} = \ln(B_0/B_t)/t$$

5   where B_0 is initially bound radioligand and B_t is bound
       radioligand remaining after t minutes dissociation.
       These values were finally expressed as % inhibition of
       the true ^{3}H-NMS dissociation rate constant (k_{off} in the
       absence of allosteric agent) and fitted to a logistic
10   function using nonlinear regression analysis.
       Theoretically the curves should have slopes of 1, and
       correspond to the occupancy curves of the allosteric
       agents at the ^{3}H-NMS-occupied receptors, regardless of
       whether the inhibition of ^{3}H-NMS dissociation is caused by
15   an allosteric change in the shape of the receptor or the
       trapping of the ^{3}H-NMS in its binding pocket by the bound
       allosteric agent (Lazareno and Birdsall, 1995).
       Initially the curve was fitted without constraints. If
       the slope factor was not different from 1, and the
20   maximal inhibition ('Emax') did not exceed about 100%,
       then the slope was constrained to 1 and the Emax was
       fitted. If the fitted Emax exceeded 100% (a physical
       impossibility, apart from experimental variation or
       error) then the Emax was constrained to 100 and the slope
25   fitted. With the compounds under study the Emax was
       often less than 100, and in most such cases the data were
       well fitted with the slope constrained to 1.

       GTPγS binding assay:
30   Membranes expressing M_1 receptors (5-20 μg/ml) were
       incubated with ^{35}S-GTPγS (0.1 nM), GDP (10^{-7}M) and ligands
       in incubation buffer in a volume of 1 ml for 30-60
       minutes at 30°C. Bound label was collected by filtration
       over glass fibre filters prewetted with water.

35

Part 1

Results

The structures of the compounds examined are shown on page 54. Figure 1 shows effects of compound 1f (staurosporine) on equilibrium ^{3}H-NMS binding at M_1 receptors in the absence and presence of a fixed concentration of ACh. ^{3}H-NMS binding was increased by staurosporine concentrations up to 10 µM and was reduced at 30 µM. The increase in ^{3}H-NMS binding reflects a decrease in the Kd of ^{3}H-NMS rather than an increase in Bmax (data not shown). The decrease in binding with 30 µM staurosporine is caused by the slowing of ^{3}H-NMS kinetics by high concentrations of staurosporine (see below) and the consequent lack of equilibration of ^{3}H-NMS binding (Lazareno and Birdsall, 1995). The effect of staurosporine on ACh binding is not clear from inspection of Figure 1, but nonlinear regression analysis of the data, which also takes into account the effects of high concentrations of staurosporine on the kinetics of ^{3}H-NMS, provided a good fit to the data (lines in Figure 1) and revealed a 4-fold negative cooperativity between ACh and staurosporine. The independent effects of staurosporine on ^{3}H-NMS and ACh binding across the four receptor subtypes are easier to visualise when the binding data are transformed into affinity ratios (Lazareno and Birdsall, 1995;Lazareno et al, 1998) (Figure 2). In theory, the EC_{50} or IC_{50} of the affinity ratio plot corresponds to the Kd of the test agent for the free receptor, and the asymptotic value corresponds to the cooperativity with the primary ligand. Staurosporine (1f) showed positive cooperativity with ^{3}H-NMS at M_1 and M_2 receptors, neutral cooperativity with ^{3}H-NMS at M_4 receptors and was inactive or neutrally cooperative at M_3 receptors. It had negative cooperativity with ACh at M_1, M_2 and M_4 subtypes and was neutral with ACh or inactive at

M_3 receptors. Staurosporine had Kd values for unoccupied receptors in the µM range (Figure 2, Table 1). In two functional assays with M_1 receptors measuring the stimulation by ACh of ^{35}S-GTPγS binding, 10 µM staurosporine reduced basal activity and the Emax by 17% ± 7% and 25% ± 4% respectively, and also caused a 2.9 ± 0.9 fold decrease in the potency of ACh, which is consistent with the 3.6-fold change predicted from the ^3H-NMS binding studies (data not shown).

Staurosporine also inhibited ^3H-NMS dissociation (Figure 3). All the curves had slope factors of 1. Staurosporine was most potent and effective at M_1 receptors, causing apparently complete inhibition of ^3H-NMS dissociation with an IC_{50} of 1 µM (Table 2). It was 3-4-fold weaker at the other receptor subtypes, and also caused submaximal inhibition of ^3H-NMS dissociation, with the smallest effect, 67% inhibition, seen at M_3 receptors. The IC_{50} values for the inhibition of ^3H-NMS. dissociation correspond in theory to the Kd values of staurosporine for the ^3H-NMS-liganded receptors, and the values at M_1 and M_2 receptors are consistent with the values predicted from the equilibrium binding studies according to the allosteric model (Table 2). There was a 2-fold disparity between predicted and observed values at M_4 receptors, probably because of inaccuracies in measuring the small degree of negative cooperativity with ^3H-NMS. In equilibrium binding studies at M_3 receptors staurosporine had little or no effect on the binding of either ^3H-NMS or ACh: the clear inhibition of ^3H-NMS dissociation caused by staurosporine over the same concentration range suggests that staurosporine was neutrally cooperative with ^3H-NMS and ACh at M_3 receptors, rather than inactive.

Gö 7874 (1i), a ring-opened analogue of staurosporine
still bearing a positive charge, showed weak negative
cooperativity with ^{3}H-NMS and stronger negative
cooperativity with ACh at M_1, M_2 and M_4 receptors, and the
5   reversed pattern at M_3 receptors (Figure 2). It was
necessary to introduce a slope factor >1 into the binding
equation for Gö 7874 in order to fit the data adequately
to the allosteric model (Table 1). Gö 7874 caused
apparently complete inhibition of ^{3}H-NMS dissociation at
10   M_1, M_2 and M_4 receptors, and submaximal inhibition at M_3
receptors. The slopes of the curves at M_1, M_2 and M_4
receptors were also >1 (Figure 3, Table 2). The ternary
complex allosteric model does not predict slope factors
different from 1, so it cannot provide a complete
15   mechanistic explanation of the data. Nevertheless, the
affinity values of Gö 7874 for the ^{3}H-NMS-occupied
receptor predicted by the model from the equilibrium
binding studies are in excellent agreement with the
observed values at M_1 and M_4 receptors (Table 2), and show
20   only a 3-fold discrepancy at M_2 and M_3 receptors, caused
possibly by a combination of inaccuracies in the
measurement of the small cooperative effects which
occurred in equilibrium studies at the M_2 and M_3 subtypes
(Table 1) and the small inhibitory effect on ^{3}H-NMS
25   dissociation from M_3 receptors.

KT5823 (1e), a ring-contracted analogue of staurosporine
in which the methylamino group is replaced by a methyl
ester, caused a large increase in ^{3}H-NMS binding at M_1 and
30   M_2 receptors, and showed neutral or small positive
cooperativity with ACh at these receptors. KT5823 was
inactive or neutrally cooperative with ^{3}H-NMS and ACh at
M_3 and M_4 receptors (Figure 2). The positive
cooperativity with NMS at M_1 receptors was confirmed in
35   functional studies in which 1mM KT5823 increased the

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potency of ACh 1.9 ± 0.9 fold at M_1 receptors for
stimulating ^{35}S-GTPγS binding, and caused a 3.3 ± 1.7 fold
increase in the affinity of unlabelled NMS (n=2, data not
shown). KT5823 inhibited ^3H-NMS dissociation completely

5   at M_1 receptors, 80% at M_2 receptors and 30-40% at M_3 and
M_4 receptors (Figure 3). The affinity of KT5823 for the
^3H-NMS-occupied receptor estimated from equilibrium
studies at M_1 and M_2 receptors was very similar to the
values measured directly. The inhibition of ^3H-NMS

10   dissociation seen at M_3 and M_4 receptors may indicate that
KT5823 is neutrally cooperative with ^3H-NMS and ACh at
these receptors, rather than inactive.

KT5720 (1a), a hexyl ester analogue of KT5823, was

15   positively cooperative with both ^3H-NMS and ACh at M_1
receptors (Figure 1, Table 1). The small (40%) increase
in ACh affinity was confirmed in more detailed assays
(Figure 5). KT5720 had little or no effect at M_3
receptors, and showed neutral cooperativity with ^3H-NMS

20   and negative cooperativity with ACh at M_4 receptors. The
effects of KT5720 at M_2 receptors are unclear: earlier
batches had small inhibitory effects with ^3H-NMS and ACh
(Figure 2), while a later batch had small positive
effects with ^3H-NMS (data not shown): no batch-dependent

25   effects were noted at the other subtypes. KT5720 caused
incomplete inhibition of ^3H-NMS dissociation at M_1, M_3 and
M_4 receptors, with little or no effect at M_2 receptors
(Figure 3). The largest effect was seen with M_1
receptors, and, at this subtype alone, low concentrations

30   of KT5720 caused a small but consistent increase in ^3H-NMS
dissociation. This phenomenon was observed in 10 out of
11 single-time point assays, with the dissociation rate
constant (k_{off}) of ^3H-NMS increased by 11 ± 1 % (n=10) in
the presence of the most effective concentration between

35   10 and 300 nM KT5720, and in two full time course studies

in which k_{off} in the presence of 0.1 µM KT5720 was
increased by 16.3 ± 0.5 % (data not shown). The affinity
of KT5720 for the ^{3}H-NMS-occupied receptor estimated from
equilibrium studies at M_1 and M_4 receptors was similar to

5   the values measured directly (Table 2).

K-252a (1b), in which the methoxy group of KT5823 is
replaced by a hydroxyl group, showed positive
cooperativity with ^{3}H-NMS at M_1 receptors and neutral or

10   small negative cooperativity with ACh (Figure 2, Table.
1). Little or no effect was seen in equilibrium binding
studies with the other subtypes. K-252a inhibited ^{3}H-NMS
dissociation at M_1 receptors, apparently by 100%. Slope
factors > 1 were required to fit the data adequately.

15   Only small, though consistent, effects on ^{3}H-NMS off rate
were seen at the other subtypes (Figure 3, Table 2).

K-252b (1c), K-252c (1g), KT-5926 (1d) and Gö 6976 (1h)
at concentrations up to 10 µM had little or no effect on

20   equilibrium binding of ^{3}H-NMS and ACh and on ^{3}H-NMS
dissociation (data not shown) and were not studied
further.

We have attempted to determine whether some of the

25   allosteric effects described above occurred through an
interaction at the same site on the receptor at which
other known allosteric agents act. Figure 5a shows the
interaction between KT5720 (1a) and gallamine on
equilibrium ^{3}H-NMS binding at M_1 receptors. Gallamine had

30   its expected inhibitory effect on ^{3}H-NMS binding, and
KT5720 showed the expected positive cooperativity with
^{3}H-NMS. If gallamine and KT5720 were acting at the same
site then gallamine should have become less potent in the
presence of KT5720 and the nonlinear regression analysis

35   would have indicated strong negative cooperativity

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between the two agents. In fact, the analysis revealed
neutral cooperativity, i.e. in equilibrium binding
studies gallamine and KT5720 interact allosterically at M_1
receptors through distinct and apparently non-interacting
sites. In similar experiments with staurosporine and
gallamine at M_1 receptors, however, there was an
negatively cooperative or competitive interaction between
the compounds (Figure 5b).

In order to study the site(s) on the M_1 receptor at which
KT5720 acts to affect ^{3}H-NMS dissociation, the
concentration-related effect of KT5720 was measured alone
and in the presence of two or three concentrations each
of gallamine, brucine and staurosporine. Very similar
results were obtained in two independent assays, and the
combined data are shown in Figure 6. The data in each
condition are shown in two forms: as percentage
inhibition of the overall control (i.e. 'true') k_{off}
measured in the absence of any test agent, and, for each
curve, as a fraction of its own control k_{off} measured in
the presence of test agent and the absence of KT5720.
This latter 'fractional effect' measure has useful
properties: if the interaction between KT5720 and the
test agent is competitive, then in the presence of test
agent the EC_{50} will increase and the asymptotic
'fractional effect' will also change; if the interaction
is noncompetitive and noninteracting (i.e. with neutral
cooperativity), and if maximal concentrations of test
agent completely inhibit ^{3}H-NMS dissociation, then in the
presence of test agent both the EC_{50} and asymptotic levels
are unchanged.

The lines in the top panel of Figure 6 (except in the
presence of staurosporine) are hyperbolic fits to the
data. The effect of low concentrations of KT5720 to

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increase ^3H-NMS dissociation was apparent in all the
curves. When the data are expressed as a fractional
effect of own control, the curves for KT5720 in the
presence of various concentrations of gallamine or
5   brucine overlap, i.e. they have the same EC_{50} and
asymptotic level. There was a small concentration-
related increase in potency in the presence of gallamine,
but this is probably experimental noise, since a
positively cooperative interaction would result in
10   decreases in the asymptotic level of the 'fraction of own
control' plots. These data therefore demonstrate that
KT5720 acts at a different site from the site(s) at which
gallamine and brucine act to inhibit ^3H-NMS dissociation
from M_1 receptors.
15

A quite different pattern of results was seen with
staurosporine. The stimulating effect of low
concentrations of KT5720 became more apparent, and the
curves tend to converge at high concentrations of KT5720
20   more than in the presence of gallamine or brucine. It
was not possible to measure EC_{50} values accurately, but
inspection of the 'fractional effect' plot suggests that
staurosporine reduced the potency of KT5720. These
results indicate that staurosporine and KT5720 compete
25   for the site which mediates inhibition of ^3H-NMS
dissociation. They strongly suggest that staurosporine
can act at different site(s) from gallamine or brucine.

Discussion

30   Five of the nine indolocarbazoles which we have studied
act allosterically at muscarinic receptors. Of these,
four have similar structures and a number of similarities
in their allosteric effects, while the fifth, Gö 7874
(1i), lacks the tetrahydrofuran/pyran ring system, which
35   may account for its somewhat different effects.

In equilibrium binding studies, the four active
staurosporine-like compounds (staurosporine (1f), KT5823
(1e), KT5720 (1a) and K-252a (1b)) showed only positive
or neutral cooperativity with ^{3}H-NMS, or were apparently

5   inactive, while positive, neutral and negative
cooperativity was observed with ACh. The four compounds
showed their highest affinity, and largest positive
effects with ^{3}H-NMS, at the M_1 receptor, while they were
inactive (or neutrally cooperative with ^{3}H-NMS and ACh) at

10   M_3 receptors. These compounds bound with slope factors of
1, except for KT5823 at M_1 receptors, and this exception
may be partly accounted for by artefacts arising from the
strong (7-10 fold) positive cooperativity with ^{3}H-NMS seen
with this compound. Gö 7874 (1i), the other positively

15   charged ligand in addition to staurosporine, also showed
selectivity for the M_1 receptor but, in contrast to the
other four compounds, it showed negative cooperativity
with ^{3}H-NMS, and both neutral and negative cooperativity
with ACh, and it bound with slope factors greater than 1.

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The four staurosporine-like compounds also showed
selectivity for the ^{3}H-NMS-occupied M_1 receptor, but this
was manifest more clearly in the magnitude of inhibition
of ^{3}H-NMS dissociation than in the affinity. Again, these

25   compounds bound to the ^{3}H-NMS-occupied receptor with
slopes of 1, except for K-252a at M_1 receptors. Gö 7874
inhibited ^{3}H-NMS dissociation completely from M_1, M_2 and M_4
receptors with slope factors significantly greater than
1.

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There seems to be a relationship between the activity of
the compounds in equilibrium binding assays and the
maximum degree of inhibition of ^{3}H-NMS dissociation: an ad
hoc correlation for the current data is that compounds

35   showing less than 50% inhibition of ^{3}H-NMS dissociation at

a particular subtype appear inactive in equilibrium
studies, while those slowing ^{3}H-NMS dissociation by > 50%
show activity in equilibrium studies. This rule works in
17/20 cases, the exceptions being staurosporine at M_3; Gö
7874 at M_3 and KT5720 at M_4 receptors. The positive
relationship between allosteric activity at equilibrium
and the degree of inhibition of ^{3}H-NMS dissociation may
reflect the degree to which binding of the allosteric
agent perturbs the primary ligand recognition site on the
receptor. Those cases where the test agent inhibits
^{3}H-NMS dissociation but appears to be inactive at
equilibrium may actually reflect a lack of cooperative
effect, i.e. neutral cooperativity, rather than a lack of
binding of the test agent at equilibrium.

According to the allosteric model, the affinity of a test
agent for the ^{3}H-NMS-occupied receptor may be estimated in
two independent ways: from direct measurement of effects
on ^{3}H-NMS dissociation, and from the product of affinity
for the free receptor and cooperativity with ^{3}H-NMS,
measured at equilibrium. In this study there are 11
instances where these measures have been determined with
sufficient precision to allow comparison. There was good
agreement between the measures: three comparisons
differed by about 3-fold, one by about 2-fold, and the
rest (7) by 60% or less, and there was no obvious bias
since in 5 cases the equilibrium estimate was larger than
the directly measured value and in 7 cases it was
smaller. These results suggest that the data can be
accounted for by the allosteric model, even though the
steep slopes seen with Gö 7874 (1i) and K-252a (1b) are
not predicted by the model.

The simple model also cannot account for the effects of
KT5720 on ^{3}H-NMS dissociation at M_1 receptors, with an

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initial speeding of dissociation by about 15 % at
submicromolar concentrations, followed by submaximal
inhibition of dissociation at higher concentrations. In
the presence of staurosporine the speeding effect became
5   more prominent, while the potency of KT5720 for slowing
^{3}H-NMS dissociation appeared to be reduced, suggesting
that KT5720 may be exerting its effects at two distinct
sites, only one of which can also be occupied by
staurosporine. In contrast, the presence of gallamine or
10   brucine had no effect on the potency of KT5270 or its
fractional asymptotic effect, suggesting that, unlike
staurosporine, gallamine and brucine act at a different
site from the site(s) by which KT5720 modulates ^{3}H-NMS
dissociation, and that there is no interaction (i.e.
15   neutral cooperativity) between the binding of KT5270 and
that of brucine or gallamine.

A similar conclusion can be drawn from equilibrium
binding studies at M_1 receptors, in which KT5720 showed no
20   interaction with gallamine. In contrast, similar
equilibrium binding studies at M_1 receptors with
staurosporine and gallamine revealed a negatively
cooperative or competitive interaction. The different
interactions with gallamine shown by staurosporine
25   (negative) and KT5720 (neutral) may be related to the
fact that staurosporine, like gallamine, is a positively
charged molecule, whereas KT5720 is neutral.

These results demonstrate that KT5720, and possibly other
30   indolocarbazoles, bind to an allosteric site on
muscarinic receptors which is distinct from the 'common
allosteric site' to which gallamine and most other
allosteric agents bind. Previously reported allosteric
agents have a positively charged nitrogen which is
35   thought to be important for their action. Staurosporine

and Gö 7874 are also positively charged, but the other
active indolocarbazoles are neutral, which suggests that
there is no necessity for a positively charged nitrogen
at this new allosteric site. The observed affinities and
5   cooperativities are sensitive to small changes in the
chemical structure of the analogues. For example,
increasing the alkyl chain length of the ester function
of K-252a or methylation of its hydroxyl group increase
affinity 3-15-fold, whereas removal of the methyl group
10   on the ester of K-252a or the alkoxy substitution of the
indolocarbazole ring generate apparently inactive
compounds.

The agents studied here are known to be potent inhibitors
15   of various protein kinases, and in most cases the agents
have much higher affinity for these targets than for
muscarinic receptors, but it is worth noting that KT5720
has only about 6-fold higher potency for its preferred
target, protein kinase A (PKA), than for the M_1 receptor
20   (log affinity of 7.2 at PKA vs. 6.4 at M_1 receptors).

One of our aims has been the development of drugs which
enhance the affinity of ACh at M_1 receptors while having
no effect on ACh binding and function at the other
25   subtypes. The detection of the allosteric properties of
KT5720 may be a step towards that goal. KT5720 was the
most potent compound at M_1 receptors with a log affinity
for the free receptor of 6.4, and it showed a small (40%)
but consistent positive cooperative effect with ACh. In
30   addition it had little or no effect on ACh affinity at
the other subtypes, so KT5720 is close to displaying an
'absolute subtype selectivity' for the M_1 receptor, i.e. a
positive or negative interaction with ACh at one receptor
subtype and neutral cooperativity at the others, so that
35   whatever concentration of agent is administered only the

one receptor subtype is affected functionally (Lazareno
and Birdsall, 1995).

5   The results in this section show a quite potent
     allosteric interactions of staurosporine and some other
     indolocarbazole analogues at muscarinic receptors which,
     at least in the case of KT5720, occur at a site distinct
     from the 'common allosteric site'. The active
     indolocarbazoles cause different maximal effects on ^{3}H-NMS
10   dissociation, and the size of the maximal effect on ^{3}H-NMS
     dissociation is a good predictor of the activity detected
     in equilibrium studies, suggesting a common mechanism for
     the two effects. In general the results from equilibrium
     and dissociation assays were mutually consistent with the
15   ternary allosteric complex model as the underlying
     mechanism of the observed effects. Finally, KT5720 is
     the most potent agent described so far showing positive
     cooperativity with ACh at M_1 receptors.

20   **Part 2**

     Results

     We also examined the interaction of a second family of
     compounds represented by general formula 2 shown on pages
     55 and 56. Figure 7 shows representative data from off
25   rate assays for these structures, and the parameter
     estimates are summarised in Table 3. Figures 8 and 9
     show representative affinity ratio plots from equilibrium
     assays, and the parameter estimates are summarised in
     Table 4.

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     2b (WIN 51708) strongly inhibited ^{3}H-NMS dissociation at
     M_2 and M_4 receptors, with about 10-fold M_4 selectivity, it
     caused submaximal inhibition at the M_1 receptor and had no
     effect at the M_3 receptor. 2c (WIN 62577), containing a
35   5-6 double bond, which reduced the affinity for the

^{3}H-NMS-occupied M$_4$ receptor by about 10-fold and led to a
smaller maximal effect at M$_2$ receptors. These effects
were reflected in the equilibrium assays, where 2b showed
a small degree of positive cooperativity with ^{3}H-NMS at M$_2$
receptors and a larger positive effect at M$_4$ receptors,
while 2c, which was up to 5-fold less potent, showed only
small negative cooperativity with ^{3}H-NMS. With respect to
ACh, 2b showed small negative cooperativity at M$_1$ and M$_3$
receptors and larger negative effects at M$_2$ and M$_4$
receptors, while 2c was also negative at M$_2$ and M$_4$
receptors, was almost neutral at M$_1$ receptors, and showed
a small (1.5-fold) positive interaction with ACh at M$_3$
receptors. This latter effect was confirmed in more
detailed assays (e.g. Figure 10). It is worth noting
that the potency and small degree of negative
cooperativity with ^{3}H-NMS of both 2b and 2c at M$_3$
receptors should result in some activity at M$_3$ receptors
in the off rate assay, whereas no activity was observed.

Removal of the bridgehead nitrogen from 2c gave 2a and
led to a 10-fold increase in affinity at M$_1$ and M$_2$
receptors, a 30-fold increase at M$_3$ receptors, but little
or no change in affinity at M$_4$ receptors. With a log
affinity of 6.5 it was the most potent of these compounds
at M$_1$ receptors. It showed 2-5 fold negative
cooperativity with both ^{3}H-NMS and ACh across the receptor
subtypes. In the off rate assay 2a had the unique effect
of speeding ^{3}H-NMS dissociation. It caused a 2-3 fold
increase in ^{3}H-NMS off rate at M$_3$ receptors, with smaller
effects at the other subtypes, with the order of
effectiveness M$_3$>M$_1$>M$_4$>M$_2$. This effect was confirmed in
full dissociation assays (Figure 11).

Replacement of the ethynyl and hydroxy groups of 2a with
a keto function, in 2f and 2i, resulted in a complete

loss of activity in the off rate assay (Figure 7) and the equilibrium assay (data not shown).

Removal of the ethynyl substituent from 2b and 2c gave
5 rise to the 17-hydroxy analogues, 2d and 2e. The compounds had slightly greater potency than their corresponding analogues in the off rate assay, and larger inhibitory effects at M_1 receptors, but still little or no effect at the ^{3}H-NMS-occupied M_3 receptor. In equilibrium
10 studies (Figure 8) 2d showed positive cooperativity with ^{3}H-NMS at all subtypes, and its log affinity of 7 at the free M_4 receptor was the most potent interaction in this study. 2e showed small negative, neutral and positive effects with ^{3}H-NMS. Both compounds had negative
15 cooperativity with ACh, except 2e which showed a small (30%) positive cooperativity with ACh at M_3 receptors.

2g and 2j are analogues of 2b lacking two rings of the steroid moiety of 2b. 2g is reported to be the trans
20 isomer and 2j is reported to be the cis isomer. 2b itself has the trans configuration, so it is very surprising that 2g was virtually inactive in the off rate assay (Figure 7) and equilibrium assays (data not shown), while 2j showed strong activity in the off rate assay
25 (albeit 10-100 fold weaker than 2b), and in equilibrium assays, where it was strongly negative with ^{3}H-NMS and ACh, and only 2-5 fold less potent than 2b.

21 is a truncated form of 2j, and no longer chiral. It
30 was also active, showing similar potency to 2j in the off rate assay (and a bigger effect on M_3 receptors), but less potency in equilibrium assays, especially at M_2 and M_4 receptors (Figure 9), leading to positive cooperativity with ^{3}H-NMS at these subtypes.

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2h is the pyrimidoimidazole analogue of 2c, lacking the
fused benzene ring. This change caused a reduction in
affinity for both free the ^{3}H-NMS-occupied receptor of 2-
20 fold, and stronger negative cooperativity with ^{3}H-NMS
5   and ACh.

2k is an analogue of 2h with the imidazole ring attached
to a different portion of the pyrimidine ring. It had a
30-200 fold higher affinity than 2h at ^{3}H-NMS-occupied
10   receptors, or more than 1000-fold at M_3 receptors if the
small effects at this subtype have been correctly
interpreted. It also had 10-50 fold higher affinity for
the free receptor than 2h, with Kd values of less than 1
mM at all subtypes. It showed strong positive
15   cooperativity with ^{3}H-NMS at M_1 receptors (Figure 9), and
was positive with ^{3}H-NMS at M_2 and M_4 receptors and weakly
negative at M_3 receptors. It had 2-4 fold negative
cooperativity with ACh.

20   2t, an analogue of 2k but with a pyrimidoimidazole
substituent on the 16,17 positions of the steroid
backbone, was inactive, reinforcing the importance of
substitution in this region for activity.

25   Most compounds which bind to the primary or allosteric
sites of muscarinic receptors have a basic nitrogen,
though neutrally charged antagonists have been described.
The two steroid structures (2m and 2n), which do not
contain nitrogen, correspond to the steroid portion of
30   2c, 2h, 2a and 2k. Surprisingly, they show activity in
both the off rate and equilibrium assays, and 2m seems to
be more potent than 2h in the off rate assay (Figure 7).
The 17-keto analogue, 2s, and the 2-substituted
analogues, 2o-2r appeared to be inactive.

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With regard to slope factors, most of the compounds had
binding slopes of 1 in equilibrium or kinetic assays. The
relatively weak compounds 21, 2j and 2h however had
steeper slopes indicating a more complex interaction.

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The effect of 2a to increase ^{3}H-NMS dissociation provides
an opportunity to assess whether 2a binds to the same
site on the ^{3}H-NMS-occupied receptor as other allosteric
agents. The dissociation rate constant (k_{off}) of ^{3}H-NMS

10   from the M_3 receptor was measured at a single time point
alone and in the presence of a range of concentrations of
2a, alone and in the presence of one or more
concentrations of a second agent. The data were
expressed as % of control k_{off}, as described in Methods,

15   and fitted to Equation 1. The results are shown in
Figure 12 and Table 6. Strychnine and gallamine reduced
the Emax of 2a, indicating that they were acting at a
different site from that occupied by 2a, but they did not
affect the EC_{50} of 2a, indicating that there was no

20   cooperative interaction, i.e. they showed neutral
cooperativity. In contrast, 1a (KT 5720), 1f
(staurosporine) and 2b (WIN 51708) did not appear to
alter the E_{max} of 2a but reduced its potency, and the data
were well fitted assuming a competitive interaction,

25   although a strongly negative interaction cannot be ruled
out. These results demonstrate that there are two
allosteric sites on the M_1 and M_3 receptors. It is worth
noting that 2b (WIN 51708) clearly binds to the ^{3}H-NMS-
occupied M_3 receptor, even though it does not modify the

30   k_{off} of ^{3}H-NMS.

Discussion
This paper describes a new series of compounds which
interact allosterically with muscarinic receptors. The

35   initial lead was provided by two commercially available

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compounds, 2b (WIN 51708) and 2c (WIN 62577). These
compounds are potent antagonists at rat NK_1 receptors, but
the affinity of these compounds, exemplified by 2b, is
reduced 400 fold at the human NK_1 receptor. In fact our
5   results show that 2b is up to 60 fold more potent on
human muscarinic receptors compared to human neurokinin
receptors.

These two compounds and the analogues described in this
10   paper exhibit positive, neutral and low negative
cooperativities with NMS and especially ACh. This latter
characteristic is important in that it suggests that the
allosteric enhancers at a specific muscarinic receptor
subtype may be synthesised in this series which are
15   neutrally cooperative with ACh (and therefore inactive at
any concentrations) at other subtypes. This form of
selectivity, based on cooperativity rather than affinity,
has been termed 'absolute subtype selectivity' (Lazareno
et al, 1998; Birdsall et al, 1999) and is a direct
20   consequence of the ternary complex allosteric model
(Lazareno and Birdsall, 1995) which underpins the
analyses of all our binding and functional data.

The ternary complex allosteric model implies that the
25   affinity of a compound for the ^3H-NMS-occupied receptor
can be estimated in two ways: as the product of affinity
for the free receptor and cooperativity with 3H-NMS from
equilibrium assays, and as the reciprocal of the IC50 (or
EC50) from off rate assays. Table 5 shows a comparison
30   between the pKocc (from equilibrium studies) and the
pKoff (from off rate studies) values of those compounds
for which there are at least two observations of each
type of measure. Of the 32 comparisons, 23 show a
discrepancy between the two measures of less than 0.3 log
35   units (2-fold), a value which is readily accounted for by

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experimental error. In most other cases the discrepancy
can be accounted for by inaccuracies either observed in
the data or predicted from the effect of the compound.
The discrepancies of 2b and 2c at M_1 receptors may be
explained by the small effects in the off rate assay, 2d
has small effects at the M_3 receptor in the off rate
assay, and at the M_4 receptor lower concentrations should
have been used to define better the parameters. 2j has
strong negative cooperativity at M_2 and M_4 receptors which
cannot be measured accurately with the experimental
designs used here, and 2k has very small effects in the
off rate assay with M_3 receptors. That leaves the 3-fold
discrepancies for both 2d and 2k at M_2 receptors, for
which there are no obvious explanations. Two outliers
out of 32 observations may be within the expected
variability of such data, and overall the data pass this
rather stringent test and are therefore consistent with
the ternary complex allosteric model as the underlying
mechanism which is responsible for effects on both
equilibrium binding and on ^3H-NMS dissociation.

2b, 2c and and the analogues examined can be considered
in simplistic terms as a fusion of a planar aromatic
heterocyclic system with an alicyclic ring system,
especially a steroid structure. Most surprisingly both
the steroid moiety alone, for example 2n, (but not some
other analogues) and the heterocyclic ring system (21)
are individually capable of interacting allosterically
with ^3H-NMS and with comparable affinities to each other.
This result implies that these compounds may interact
with different but contiguous subdomains of the same
pharmacophore.

Binding to the allosteric site is sensitive to the nature
of the heterocycle when the steroid ring is kept constant

(compare 2h, 2a and 2k with 2c). Equally the nature of the 17-substituent on the steroid ring seems important in the fused systems. The analogues with a 17-keto function appear inactive, whereas all compounds with a 17β

5   hydroxyl group are active. The presence of the 17-α ethynyl group has more subtle effects (mainly on the M_4 receptor) and these seem to be interrelated with effects of the saturation status of the 5-6 bond.

10   Another surprising result is that 2j is active whereas 2g appears to be inactive. Both these compounds are racemates and they represent truncated analogues of 2b with the AB rings of the steroid in the cis and trans configurations respectively. Activity was expected to be

15   associated with the trans isomer rather than the cis isomer.

       Many of the compounds investigated in this study, in contrast to most muscarinic allosteric agents, do not

20   inhibit the association and dissociation of ^{3}H-NMS completely at high concentrations. They often only produce a 2-fold or less slowing effects on the kinetics and in some instances, especially at M_3 receptors, very small effects indeed (Figure 7).

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       The remarkable finding is that 2a increases the dissociation rate of ^{3}H-NMS with the largest effect being observed at M_3 receptors and the smallest effect at M_2 receptors. It is noteworthy that the data from

30   equilibrium and off rate studies with 2a are entirely consistent with the allosteric model, i.e. there are no discrepancies between estimates of affinity for the ^{3}H-NMS-occupied receptor from the two types of assay, and the slope factors were 1 or close to 1. The agreement

35   occurs despite the enhancement of ^{3}H-NMS dissociation by

2a being the opposite to the inhibitory effect seen with all the other compounds in this study, and in every other published study of allosteric agents at muscarinic receptors.

This unique effect of 2a allowed us to assess whether other allosteric agents inhibit ^{3}H-NMS dissociation by acting at the same site as 2a. We found that 1a (KT5720), 1f (staurosporine) and 2b bind to the same site as 2a on the ^{3}H-NMS-liganded receptor, but gallamine and strychnine bind to a different site and have no effect on the binding of 2a, i.e. gallamine and strychnine show neutral cooperativity with 2a.

In conclusion, the following general points can be made. (1) allosteric agents can enhance, inhibit, or have no effect on the dissociation rate of ^{3}H-NMS. (2) There are at least two nonoverlapping allosteric sites, the 'common' site and the 'WIN' site; (3) Both allosteric sites can support a positive cooperative interaction with ACh; (4) The use of 2a provides a test of whether another allosteric agent binds to the 'WIN' site.

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The references mentioned herein are all expressly incorporated by reference.

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		R1	R2	R3
1a	KT5720	OH	$COO(CH_2)_5CH_3$	H
1b	K-252a	OH	$COOCH_3$	H
1c	K-252b	OH	COOH	H
1d	KT5926	OH	$COOCH_3$	$O(CH_2)_2CH_3$
1e	KT5823	OCH_3	$COOCH_3$	H
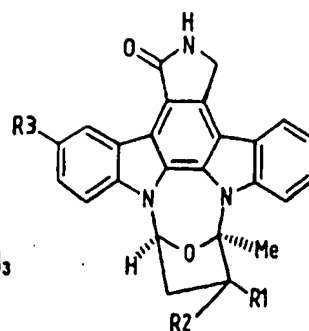
1f   Staurosporine
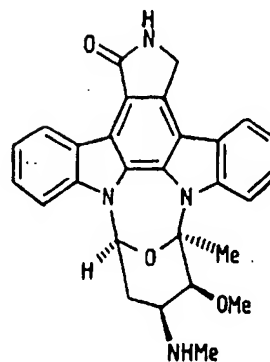
		R1	R2
1g	K-252c	H	H
1h	Gö 6976	CH_3	CH_2CH_2CN
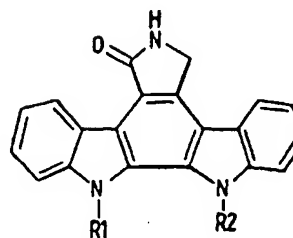
1i   Gö 7874 HCl
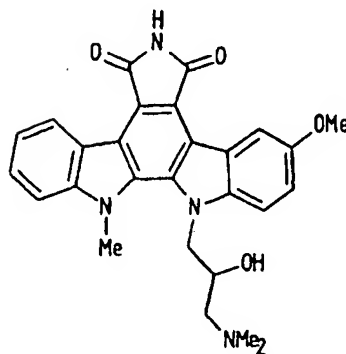
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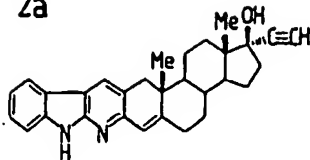
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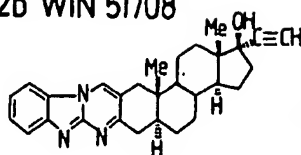
2c WIN 62577
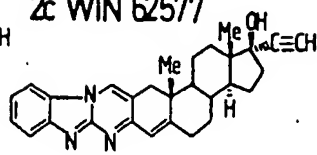
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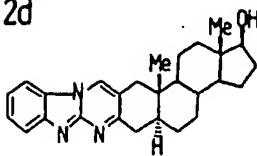
2e
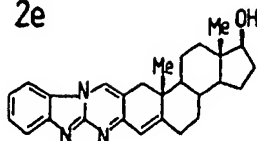
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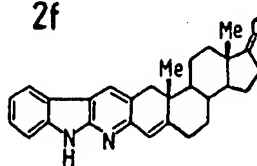
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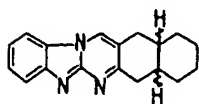
2h

2i

2j cis
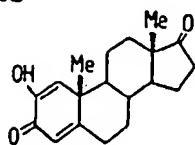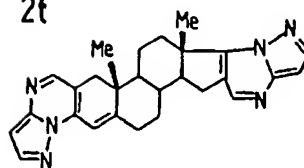
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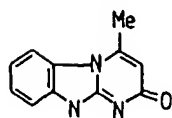
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2n

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2o

2p

2q

2r

2s

2t

2u

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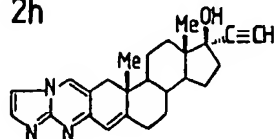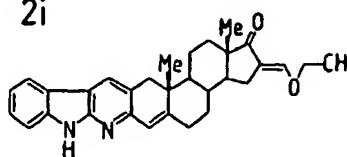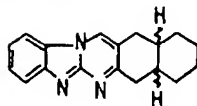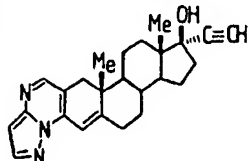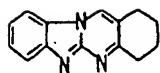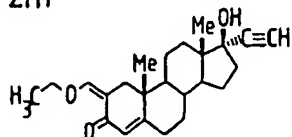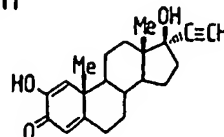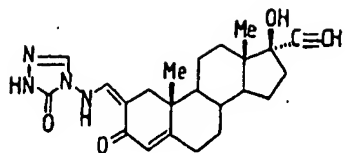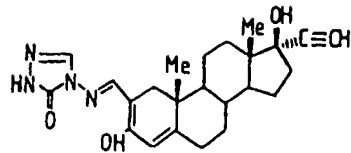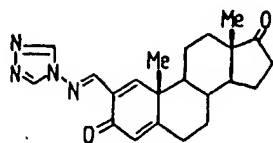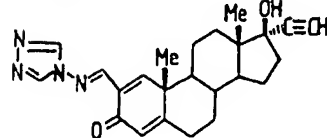
Table 1

Equilibrium binding parameters of indolocarbazoles with ^{3}H-NMS and Ach at muscarinic receptors. Assays such as those shown in Figures 2 and 5 were fitted to Equation 1 or 2 as appropriate (see Methods). The results are from at least 3 assays, except * n=2. Empty cells indicate that it was not possible to obtain at least 2 sets of parameter estimates.

#Some, but not all, of the values were obtained from analyses in which the affinity for the ^{3}H-NMS occupied receptor was fixed at the mean value obtained from offrate assays and shown in Table 2.

name			pK	slope	M_1 cooperativity ^{3}H-NMS	ACh	pK	slope	M_2 cooperativity ^{3}H-NMS	ACh
1a	KT 5720	mean	6.42*	1.00	1.94	1.39	5.29	1.05	1.56	0.82
		sem	0.09	0.00	0.14	0.09	0.33	0.05	0.60	0.45
1b	K-252a	mean	5.13**	1.00	2.62	0.86				
		sem	0.14	0.00	0.68	0.13				
1e	KT 5823	mean	5.70	1.44	8.11	1.31	5.67*	1.00	3.27	1.34
		sem	0.05	0.14	1.61	0.44	0.15	0.00	0.19	0.03
1f	staurosporine	mean	5.91	1.01	1.53	0.19	5.13	1.00	1.49	0.42
		sem	0.03	0.01	0.06	0.04	0.05	0.00	0.06	0.04
1i	Go 7874	mean	5.77*	1.60	0.54	0.07	5.03*	1.91	0.66	0.24
		sem	0.16	0.17	0.04	0.00	0.16	0.08	0.00	0.11

name			pK	slope	M_3 cooperativity ^{3}H-NMS	ACh	pK	slope	M_4 cooperativity ^{3}H-NMS	ACh
1a	KT 5720	mean					6.42*	1.00	0.92	0.55
		sem					0.25	0.00	0.01	0.08
1b	K-252a	mean								
		sem								
1e	KT 5823	mean								
		sem								
1f	staurosporine	mean					5.31	1.00	0.88	0.15
		sem					0.09	0.00	0.02	0.03
1i	Go 7874	mean	5.12*	1.50	0.32	0.72	5.71*	1.47	0.80	0.10
		sem	0.01	0.50	0.09	0.05	0.13	0.21	0.05	0.00

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Table 2

% inhibition of ^{3}H-NMS dissociation from muscarinic receptors by indolocarbazoles. Curves such as those shown in Figure 4 were fitted to a logistic equation as described in Methods. The 'estd pK' is the product of affinity for the free receptor and cooperativity with ^{3}H-NMS derived from the equilibrium binding assays summarised in Table 1. The '*diff*' is the difference between the observed pK (-log IC$_{50}$) and 'estd pK'. Empty cells indicate that it was not possible to obtain at least 2 sets of parameter estimates. The ^{3}H-NMS dissociation rate constants (minutes^{-1}) observed in this study are (mean ± sem (n)): M$_1$ 0.058 ± 0.002 (26); M$_2$ 0.34 ± 0.01 (12); M$_3$ 0.054 ± 0.002 (10); M$_4$ 0.057 ± 0.002 (10).

name		M$_1$					M$_2$				
		pK	slope	Emax	estd pK	*diff*	pK	slope	Emax	estd pK	*diff*
1a KT 5720	mean	6.18	1.00	56.79	6.70	-0.52				5.36	
	sem	0.15	0.00	3.81	0.09					0.26	
	n	7			6					5	
1b K-252a	mean	5.54	1.40	100.00			5.65	1.00	35.05		
	sem	0.01	0.07	0.00			0.00	0.00	2.35		
	n	2					2				
1c KT 5823	mean	6.40	1.00	103.55	6.58	-0.18	6.21	1.00	77.85	6.18	0.02
	sem	0.01	0.00	3.15	0.08		0.05	0.00	1.95	0.18	
	n	2			4		2			2	
1f staurosporine	mean	6.01	1.00	104.73	6.10	-0.09	5.40	1.00	90.77	5.31	0.09
	sem	0.08	0.00	5.28	0.04		0.02	0.00	8.34	0.06	
	n	3			5		3			3	
1i Go 7874	mean	5.70	1.34	100.00	5.50	0.19	5.30	1.31	100.00	4.85	0.45
	sem	0.02	0.01	0.00	0.19		0.01	0.01	0.00	0.16	
	n	2			2		2			2	

name		M$_3$					M$_4$				
		pK	slope	Emax	estd pK	*diff*	pK	slope	Emax	estd pK	*diff*
1a KT 5720	mean	6.66	1.00	38.71			6.33	1.00	24.70	6.38	-0.05
	sem	0.15	0.00	1.52			0.08	0.00	0.21	0.24	
	n	3					3			2	
1b K-252a	mean	5.55	1.00	37.10			5.37	1.00	48.95		
	sem	0.00	0.00	4.20			0.02	0.00	5.75		
	n	2					2				
1c KT 5823	mean	6.45	1.00	29.80			5.88	1.00	41.50		
	sem	0.08	0.00	5.70			0.04	0.00	4.40		
	n	2					2				
1f staurosporine	mean	5.48	1.00	67.10			5.61	1.00	88.00	5.25	0.36
	sem	0.07	0.00	2.77			0.08	0.00	6.20	0.08	
	n	3					3			3	
1i Go 7874	mean	5.05	1.00	39.50	4.60	0.45	5.71	1.64	100.00	5.60	0.11
	sem	0.28	0.00	14.00	0.15		0.03	0.35	0.00	0.10	
	n	2			2		2			2	

Table 3

Parameter estimates from offrate assays. Emax indicates the maximal % inhibition of ^{3}H-NMS dissociation. Empty cells indicate that at least two reliable estimates were not obtained. Estimates could not be obtained for 2f, 2g and 2i. n>=3, except * n=2, # n=1

Name		M$_1$ pK	slope	Emax	M$_2$ pK	slope	Emax	M$_3$ pK	slope	Emax	M$_4$ pK	slope	Emax
2a	987 mean	6.10	1	-91.90	5.81	1	-34.30	6.08	1	-146.35	6.04	1	-66.03
	sem	0.04	0	10.80	0.19	0	12.24	0.06	0	17.99	0.09	0	8.51
2b WIN 51708	690 mean	5.55	1	49.71	5.93	1	94.66				6.78	1	95.78
	sem	0.12	0	3.51	0.02	0	1.16				0.06	0	4.01
2c WIN 62577	691 mean	5	1	64.89	5.31	1.29	65.90				5.63	1	79.39
	sem	0.22	0	23.48	0.15	0.29	10.90				0.06	0	3.82
2d	924 mean	5.85	1.11	92.25	6.08	0.97	102.29	6.10*	1	29.79	6.98	1	101.79
	sem	0.05	0.12	4.36	0.03	0.03	2.26	0.12	0	6.09	0.03	0	3.12
2e	923 mean	5.32	1	88.52	5.58	0.99	93.12				6.19	1.02	97.40
	sem	0.10	0	1.74	0.01	0.01	3.26				0.04	0.01	1.37
2h	986 mean	4.68	1.77	101.67	4.34	1.62	100	3.50	1	100	4.63	1.37	99.63
	sem	0.04	0.08	1.67	0.09	0.09	0	0.02	0	0	0.08	0.12	0.37
2j cis	926 mean	4.71*	1.44	100	4.51*	1.02	101.10	4.72*	1	48.58	4.73*	1.53	87.83
	sem	0.08	0.09	0	0.05	0.02	1.10	0.01	0	3.64	0.12	0.53	15.37
2k	988 mean	6.86	0.99	101.02	6.21	1.06	94.52	6.71	1	11.21	6.30	1	91.50
	sem	0.04	0.01	1.46	0.05	0.06	1.99	0.16	0	1.47	0.07	0	3.24
2l	925 mean	4.77	1.79	99.78	4.84	1.68	103.67	4.60	1.77	100.77	4.78	1.81	100.91
	sem	0.02	0.08	0.22	0.01	0.07	1.88	0.02	0.06	0.77	0.04	0.07	1.59
2m	983 mean	5.17#	1	31.42	4.26#	1	105.20	4.92#	1	40.17	4.95#	1	90.54
	sem												
2n	982 mean				4.33	1	93.76	5.35	1	12.96	4.24	1.39	100
	sem				0.09	0	9.85	0.56	0	1.53	0	0.04	0

Table 4

Parameter estimates from equilibrium assays.

PK is the estimate of the log affinity of the agent for the free receptor.

'alloN' and 'alloA' are the estimates of cooperativity with ^{3}H-NMS and Ach respectively.

Estimates could not be obtained for 2f, 2g and 2i.

n>=3, except * n=2, # n=1

Name		M1 pK	M1 slope	M1 alloN	M1 alloA	M2 pK	M2 slope	M2 alloN	M2 alloA	M3 pK	M3 slope	M3 alloN	M3 alloA	M4 pK	M4 slope	M4 alloN	M4 alloA
2a	987 mean	6.48	1.11	0.28	0.50	6.29	1.01	0.55	0.14	6.60	1.37	0.29	0.53	6.12	1.26	0.45	0.27
	sem	0.07	0.13	0.02	0.04	0.10	0.01	0.02	0.05	0.06	0.16	0.01	0.04	0.09	0.26	0.01	0.05
2b WIN 51708	690 mean	5.77	1.24	0.25	0.44	5.93	1.22	1.70	0.22	5.46	1.33	0.46	0.74	6.18	1	3.12	0
	sem	0.07	0.14	0.04	0.07	0.06	0.22	0.21	0.01	0.13	0.33	0.09	0.17	0.03	0	0	0
2c WIN 62577	691 mean	5.51	1	0.20	0.70	5.25	0	0.62	0.24	5.13	1.40	0.38	1.78	5.90	1	0.79	0.08
	sem	0.08	0	0.06	0.08	0.28	0	0.13	0.13	0.17	0.24	0.14	0.50	0.17	0	0.05	0.04
2d	924 mean	5.67	0.73	1.74	0.26	8.55	0.87	1.33	0.36	5.28	1	1.79	0.37	7.02	0.64	2.16	0.16
	sem	0.18	0.14	0.26	0.09	0.20	0.13	0.10	0.08	0.29	0	0.30	0.08	0.11	0.36	0.21	0.06
2e	923 mean	5.68	1	0.60	0.35	5.57	1	0.73	0.15	4.87	1	0.37	1.27	5.88	0.83	1.61	0.08
	sem	0.03	0	0.04	0.02	0.11	0	0.09	0.09	0.36	0	0.20	0.21	0.14	0.09	0.37	0.03
2h	986 mean	4.81	1.33	0.26	0.15	4.79	1.04	0.32	0.02	4.52	1.42	0	0.12	4.99	1.19	0.28	0.01
	sem	0.13	0.42	0.26	0.13	0.02	0.04	0.01	0.02	0.06	0.27	0	0.01	0.08	0.22	0.12	0.01
2j cls	926 mean	5.26	1.23	0.04	0.18	5.27	1.35	0.07	0.03	5.12	1.58	0.02	0.23	5.52	1.91	0.12	0.01
	sem	0.02	0.29	0.04	0.16	0.06	0.09	0.05	0.02	0.01	0.23	0.02	0.11	0.03	0.09	0	0.01
2k	988 mean	6.02	0.79	8.13	0.38	6.57	1.03	1.37	0.46	6.26	1	0.35	0.40	6.13	0.85	1.52	0.41
	sem	0.14	0.21	1.53	0.12	0.15	0.28	0.07	0.01	0.05	0	0.09	0.07	0.05	0.15	0.08	0.02
2l	925 mean	4.72	2	0.79	0.08	4.50	2.01	1.71	0.02	4.81	1.66	0.60	0.02	4.62	1.33	1.18	0.06
	sem	0.01	0	0.15	0.01	0.09	0.01	0.17	0.02	0.07	0.09	0.07	0.02	0.05	0.33	0.09	0.02
2m	983 mean	4.57*	1	0	0.23	4.96*	1	0.87	0.35	3.90*	1	0	0.20	4.57*	1	1.48	0.03
	sem					0.36				0.22							
2n	982 mean	4.31	1	0	0.40	4.95	1	1.02	0.56	4.13	1	0.28	0.57	4.47	1	0.76	0.12
	sem	0.09	0	0	0.09	0.36	0	0.03	0.08	0.22	0	0.28	0.05	0.12	0	0.09	0.12

Table 5

Comparison of affinity estimates at ^{3}H-NMS-occupied receptors from equilibrium (pKocc) and offrate (pKoff) assays. '*diff*' is the difference between pKocc and pKoff. n>=2

	Name	M$_1$ pKocc	sem	pKoff	sem	diff	M$_2$ pKocc	sem	pKoff	sem	diff
2a	987	5.92	0.09	6.10	0.04	-0.18	6.03	0.12	5.81	0.19	0.22
2b	WIN 51708	5.14	0.13	5.55	0.12	-0.41	6.15	0.06	5.93	0.02	0.22
2c	WIN 62577	4.63	0.37	5	0.22	-0.37	5.02	0.36	5.31	0.15	-0.29
2d	924	5.90	0.16	5.85	0.05	0.05	6.67	0.23	6.08	0.03	0.59
2e	923	5.46	0.05	5.32	0.10	0.13	5.43	0.17	5.58	0.01	-0.16
2h	986						4.29	0.04	4.34	0.09	-0.05
2j cis	926						3.97	0.32	4.51	0.05	-0.55
2k	988	6.91	0.06	6.86	0.04	0.05	6.70	0.12	6.21	0.05	0.49
2l	925	4.60	0.08	4.77	0.02	-0.16	4.73	0.05	4.84	0.01	-0.11
2n	982						4.96	0.35	4.33	0.09	0.63

	Name	M$_3$ pKocc	sem	pKoff	sem	diff	M$_4$ pKocc	sem	pKoff	sem	diff
2a	987	6.06	0.04	6.08	0.06	-0.03	5.77	0.09	6.04	0.09	-0.27
2b	WIN 51708						6.67	0.07	6.78	0.06	-0.10
2c	WIN 62577						5.80	0.18	5.63	0.06	0.17
2d	924	5.52	0.22	6.10	0.12	-0.57	7.35	0.15	6.98	0.03	0.37
2e	923						6.06	0.05	6.19	0.04	-0.13
2h	986						4.40	0.28	4.63	0.08	-0.22
2j cis	926						3.85	0.02	4.73	0.12	-0.88
2k	988	5.77	0.18	6.71	0.16	-0.94	6.31	0.06	6.30	0.07	0.01
2l	925	4.58	0.12	4.60	0.02	-0.02	4.69	0.09	4.78	0.04	-0.09
2n	982						4.34	0.17	4.24	0	0.10

Table 6

Estimates of log affinity values for the ^{3}H-NMS-occupied M$_3$ receptor and maximal % inhibition of ^{3}H-NMS dissociation rate, derived from competition assays with 2a analysed with the allosteric model (Equation 1 in Appendix 2). The cooperativity values were fixed at 0 (competitive or strong negative cooperative interaction) or 1 (neutral cooperativity, noninteracting). Slope values for the test agent were fixed at 1, except for staurosporine (slope=1.31 ± 0.16, n=3).

	Name	n	pK	Emax %	cooperativity
	gallamine	2	3.38 ± 0.02	100	1
	strychnine	4	4.06 ± 0.01	100	1
	KT 5720	3	6.11 ± 0.10	50 ± 4	0
	staurosporine	3	5.79 ± 0.17	43 ± 1	0
2b	WIN 51708	3	5.78 ± 0.06	0 ± 20	0
2k	988	2	no effect		

Claims:

1.   A method for aiding in the identification of
compounds capable of modulating the binding of a primary
ligand to a muscarinic receptor by binding to an
allosteric site of the muscarinic receptor which is
capable of binding to compound 1a and/or compound 2a, the
method comprising:

    (a)   contacting the muscarinic receptor and the
primary ligand with one or more concentrations of a
candidate compound; and,

    (b)   determining whether the candidate compound
modulates the binding of a primary ligand to the
muscarinic receptor by binding to the allosteric site of
the receptor which is capable of binding compound 1a
and/or compound 2a.

2.   The method of claim 1 wherein brucine, gallamine or
strychnine do not substantially bind to the allosteric
site.

3.   The method of claim 1 or claim 2, wherein the
muscarinic receptor is a human M_1, M_2, M_3, M_4 or M_5
muscarinic receptor.

4.   The method of any one of claims 1 to 3, wherein the
candidate compound is selected if it enhances the binding
of the primary ligand to the muscarinic receptor.

5.   The method of any one of claims 1 to 4, wherein the
candidate compound is selected if it reduces the binding
of the primary ligand to the muscarinic receptor.

6.   The method of claim any one of claims 1 to 5,
wherein the method is repeated with different muscarinic
receptor subtypes.

7.   The method of claim 6, wherein candidate compound is selected if it binds the allosteric site and has no effect on the binding of the primary ligand to one or more of the muscarinic receptor subtypes but enhances or
5   reduces the binding of the primary ligand at other muscarinic receptor subtype or subtypes.

8.   The method of claim 1, wherein the candidate compound is selected if it changes the dissociation rate
10   of the primary ligand from the muscarinic receptor or changes the ability of an allosteric ligand to affect the dissociation rate of the primary ligand from the muscarinic receptor.

15   9.   The method of claim 8, wherein the allosteric ligand is capable of binding to the common allosteric site of the muscarinic receptor.

10.   The method of any one of claims 1 to 9, wherein the
20   binding of the candidate compound to the allosteric site is determined in assays employing two primary ligands which compete for the primary ligand binding site, one of which is labelled.

25   11.   The method of claim 10, wherein the labelled primary ligand is NMS and the other primary ligand is ACh.

12.   The method of any one of claims 1 to 11, wherein the method employs the muscarinic receptor, a candidate
30   compound and a primary ligand, in the presence or absence of one or more concentrations of a further allosteric ligand.

13.   The method of any one of claims 1 to 11, wherein the
35   method determines the binding of a candidate compound to

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the allosteric site using labelled primary ligand in
assays which determine the primary ligand dissociation
rate constant in the presence and absence of one or more
concentrations of the candidate compound.

14.   The method of claim 13, wherein the primary ligand
is NMS.

15.   The method of any one of claims 1 to 14, further
comprising quantitating the effects of a test compound,
which has been demonstrated in the general assays to be
allosteric, on the equilibrium allosteric effects of
ligands which are known to bind one or other of the two
allosteric sites.

16.   The method of any one of claims 1 to 15, wherein the
primary ligand is acetylcholine (ACh) or N-
methylscoplolamine (NMS), or another appropriate
competitive muscarinic agonist or antagonist.

17.   A method which comprises, having identified a
candidate compound by the method of any one of claims 1
to 16, the further step of formulating the compound as a
pharmaceutical composition.

18.   Use of a compound as obtainable by the method of any
one of claims 1 to 16 for the preparation of a medicament
for the treatment of a conditions mediated by the binding
of the primary ligand to the muscarinic receptor.

19.   A method of modulating the response of a muscarinic
receptor to a primary ligand, the method comprising
contacting the muscarinic receptor with a compound which
binds to an allosteric site of the muscarinic receptor
which is capable of binding to compound 1a and/or 2a and

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which thereby modulates the binding of the primary ligand
to the muscarinic receptor.

20.   Use of a compound for the preparation of a
medicament for the treatment of a condition mediated by
the binding of a primary ligand to a muscarinic receptor,
wherein the compound binds to an allosteric site of the
muscarinic receptor which is capable of binding to
compound 1a and/or 2a and thereby modulates the binding
of the primary ligand to the muscarinic receptor.

21.   The use of claim 20, wherein the compound is
represented by general formula 1 or 2.

22.   The use of claim 20 or claim 21, wherein the primary
ligand is acetylcholine (ACh) or N-methylscopolamine
(NMS).

23.   The use of any one of claims 20 to 22, wherein the
condition is Alzheimer's disease, Parkinson's disease,
motion sickness, Huntingdon's chorea, schizophrenia,
depression, anxiety, sedation, analgesia, stroke,
preanaesthetic, antispasmodic, irritable bowel syndrome,
bladder-incontinence or retention, peptic ulcer disease,
bronchitis/asthma/chronic obstructive airway disease,
sinus bradycardia, cardiac pacemaker regulation,
glaucoma, achalasia, symptomatic diffuse oesophageal
spasm, biliary dyskinesia, scleroderma, diabetes
mellitus, lower oesophageal incompetence, intestinal
pseudo obstruction, regulation of sleep, control of pupil
diameter or non-ulcer dyspepsia.

24.   The use of any one of claims 20 to 23, wherein the
binding of the compound to the allosteric site enhances
the binding of the primary ligand to the muscarinic

receptor.

25. The use of any one of claims 20 to 23, wherein the
binding of the compound to the allosteric site reduces
the binding of the primary ligand to the muscarinic
receptor.

26. The use of any one of claims 20 to 23, wherein the
binding of the compound to the allosteric site has no
effect on the binding of the primary ligand to one or
more of the muscarinic receptor subtypes (neutral
cooperativity) but has an allosteric effect (positive or
negative cooperativity) at other subtype or subtypes.

27. The use of any one of claims 20 to 26, wherein the
allosteric site does not bind brucine, gallamine or
strychnine.

28. The use of any one of claims 20 to 27, wherein the
muscarinic receptor is a human M_1, M_2, M_3, M_4 or M_5
muscarinic receptor.

29. Use of an allosteric site of a muscarinic receptor
which is capable of binding to compound 1a and/or 2a in
screening for compounds which are capable of modulating
the binding of a primary ligand to a muscarinic receptor
by binding to the allosteric site.

30. A compound represented by general formula 1 or 2 for
use in a method of medical treatment.

31. The compound of claim 30, wherein the compound is an
allosteric agent which modulates the binding of a primary
ligand to a muscarinic acetylcholine receptor.

1/23
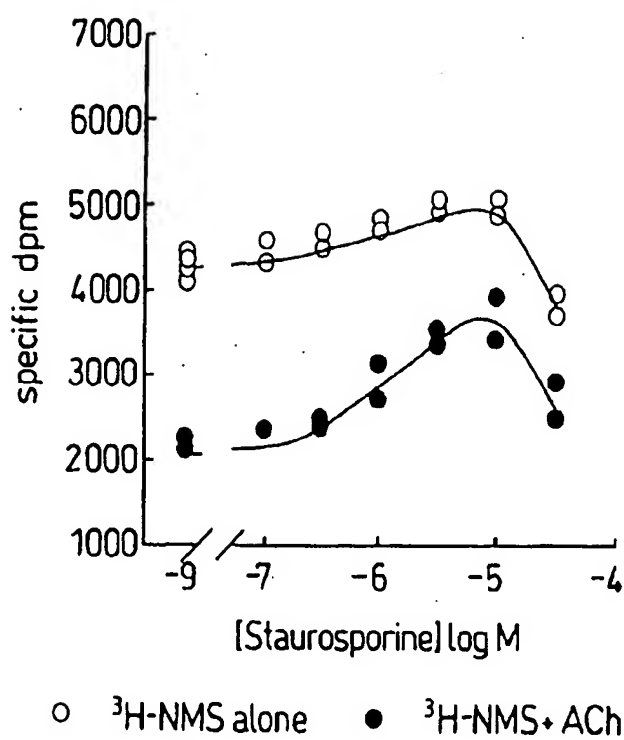
Fig. 1

Fig. 2 (part 1 of 3)

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Fig. 2 (part 2 of 3)

Fig. 2 (part 3 of 3)

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Fig. 3

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Fig. 4

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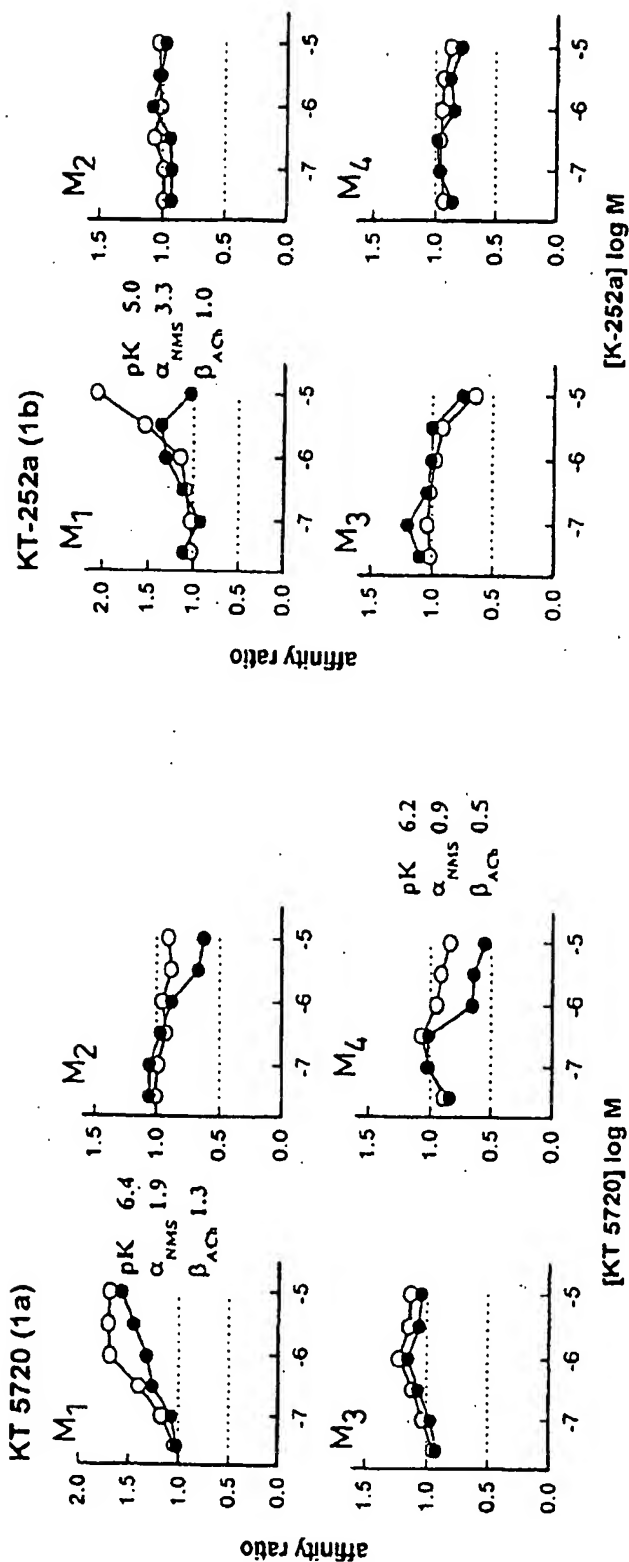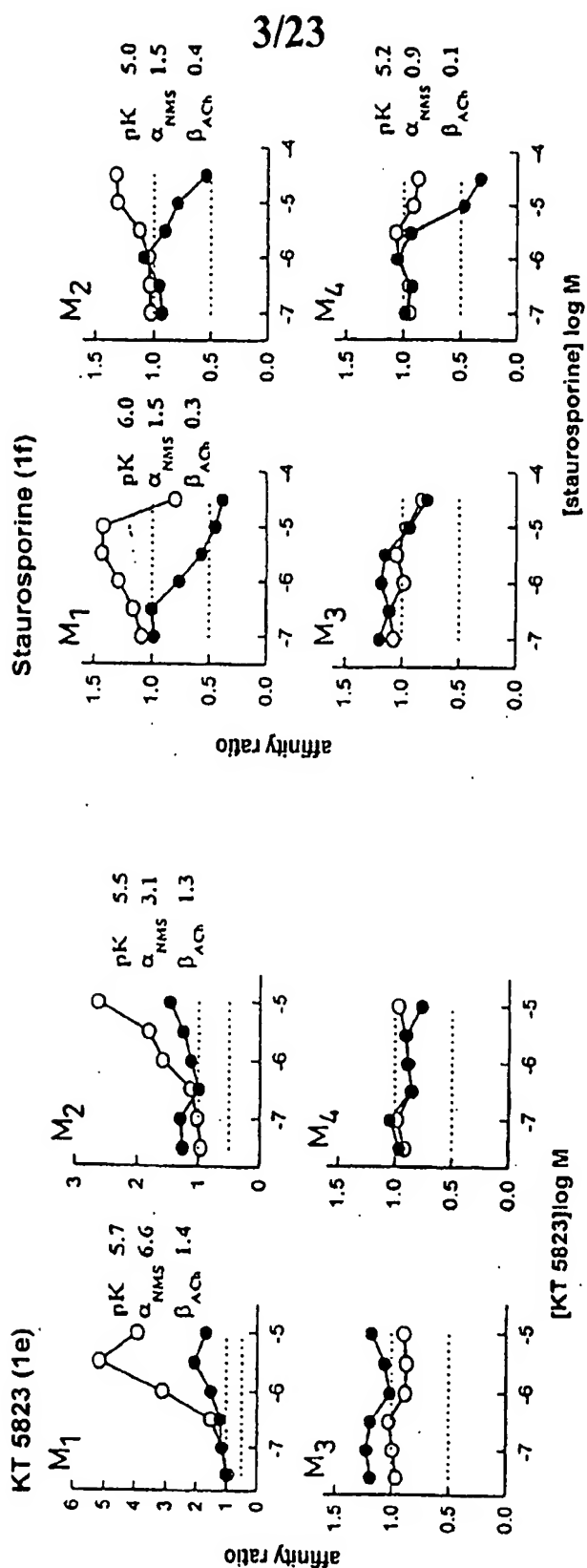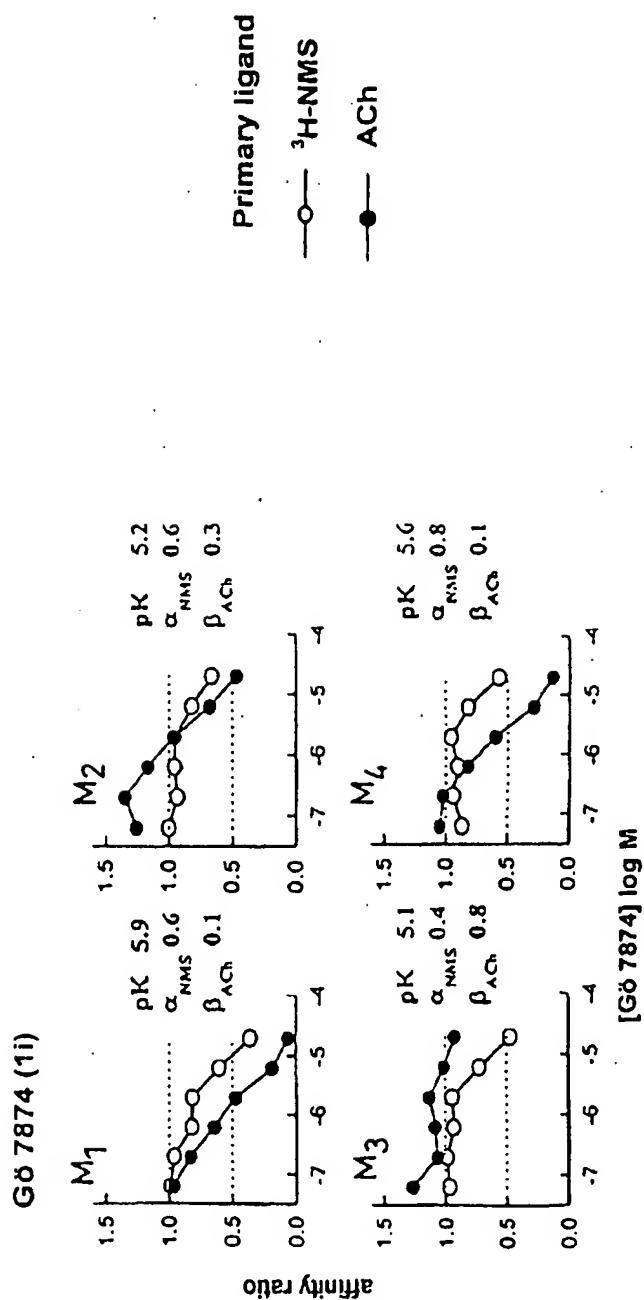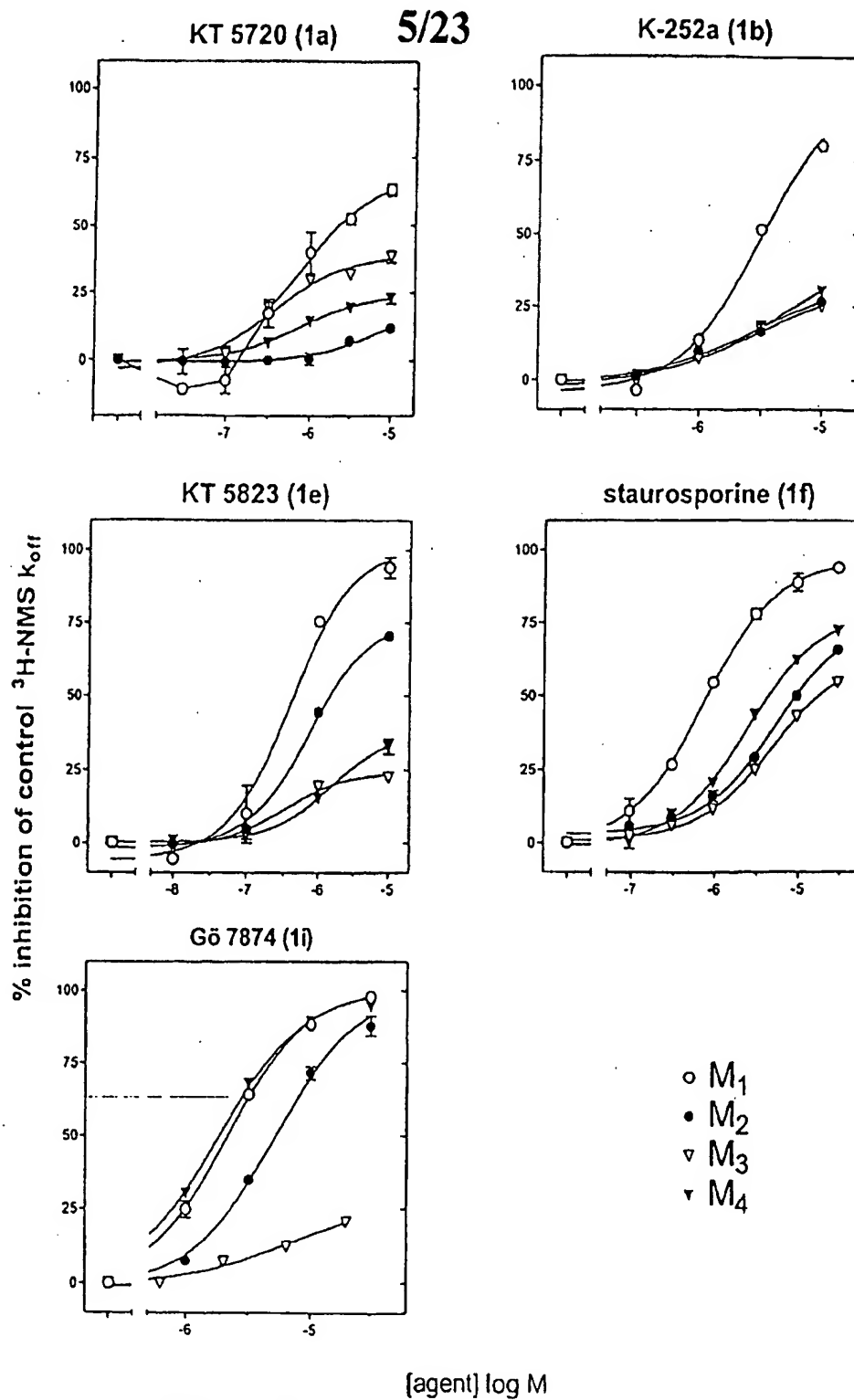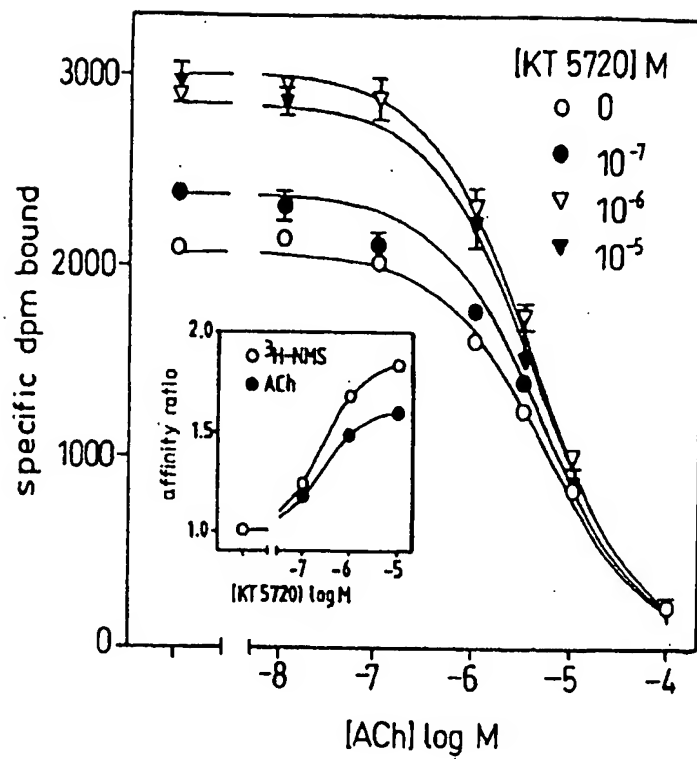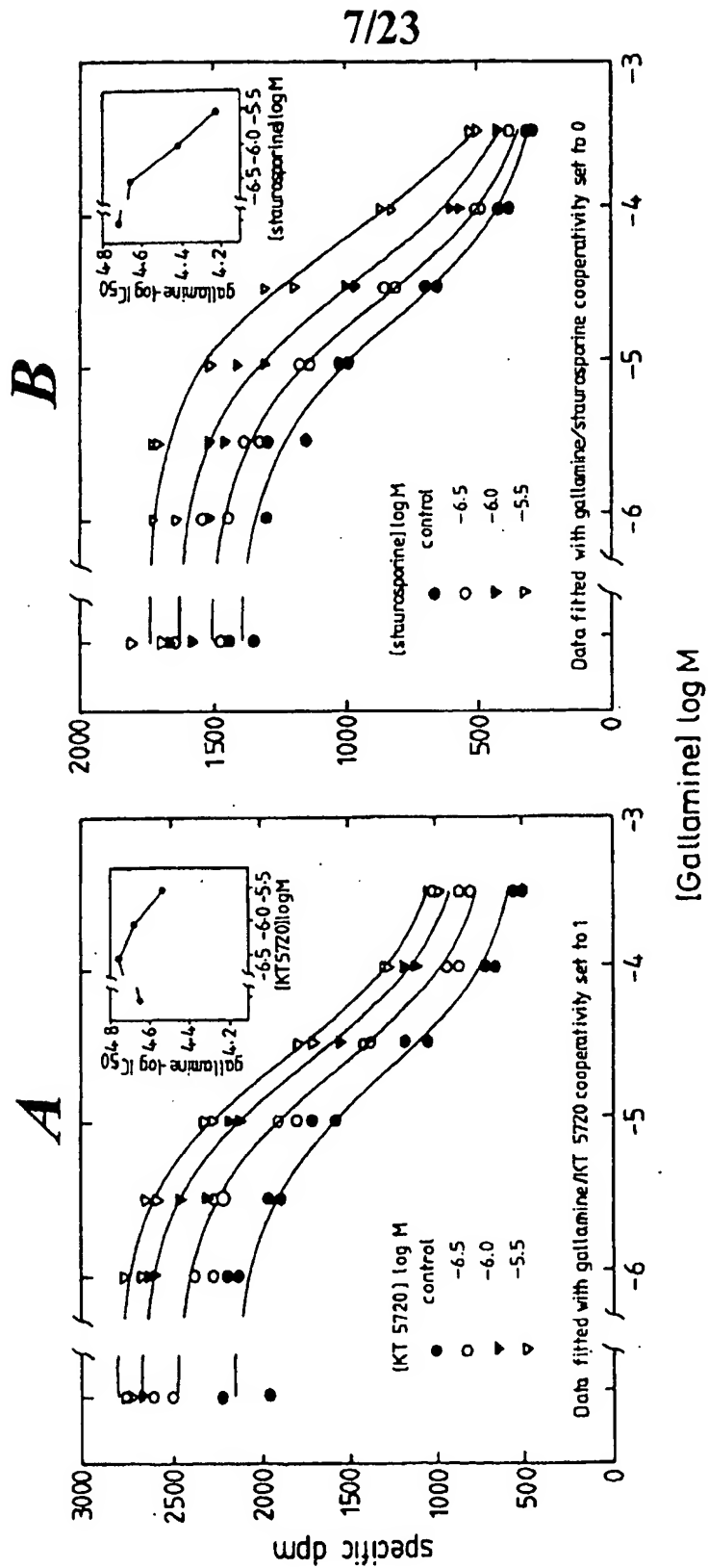
Fig. 5

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Fig. 6 (part 1 of 2)
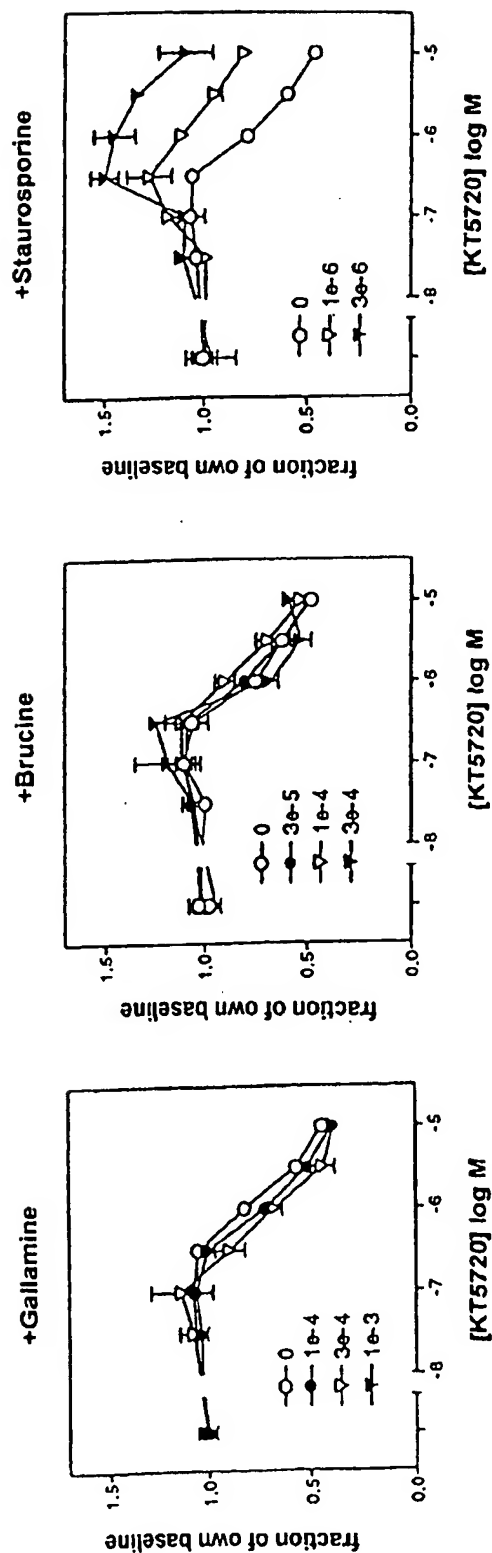
Fig. 6 (part 2 of 2)

Fig. 7 (part 1 of 3)

Fig. 7 (part 2 of 3)

Fig. 7 (part 3 of 3)

2a

Primary ligand

—○— ^{3}H-NMS

—●— ACh
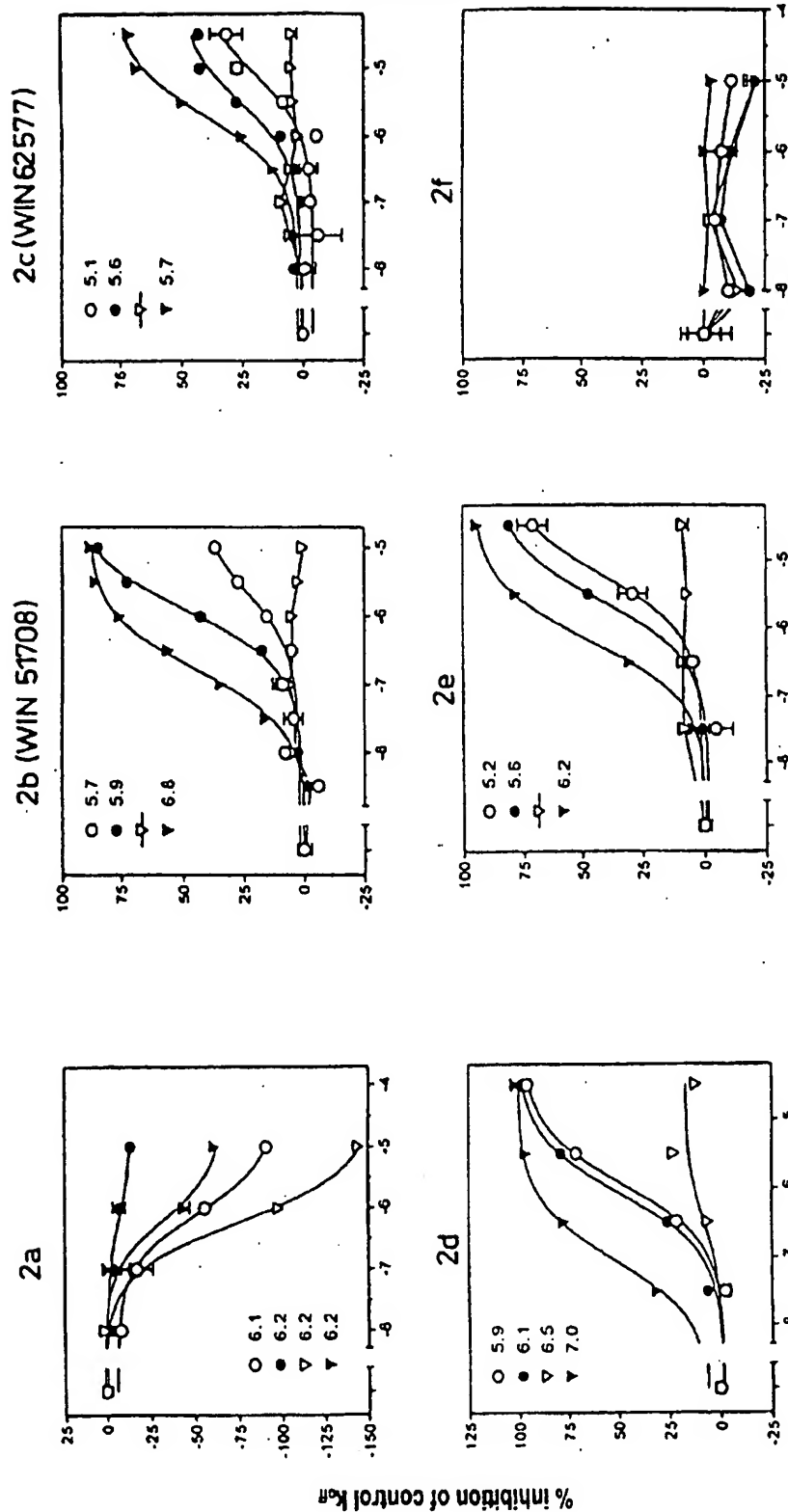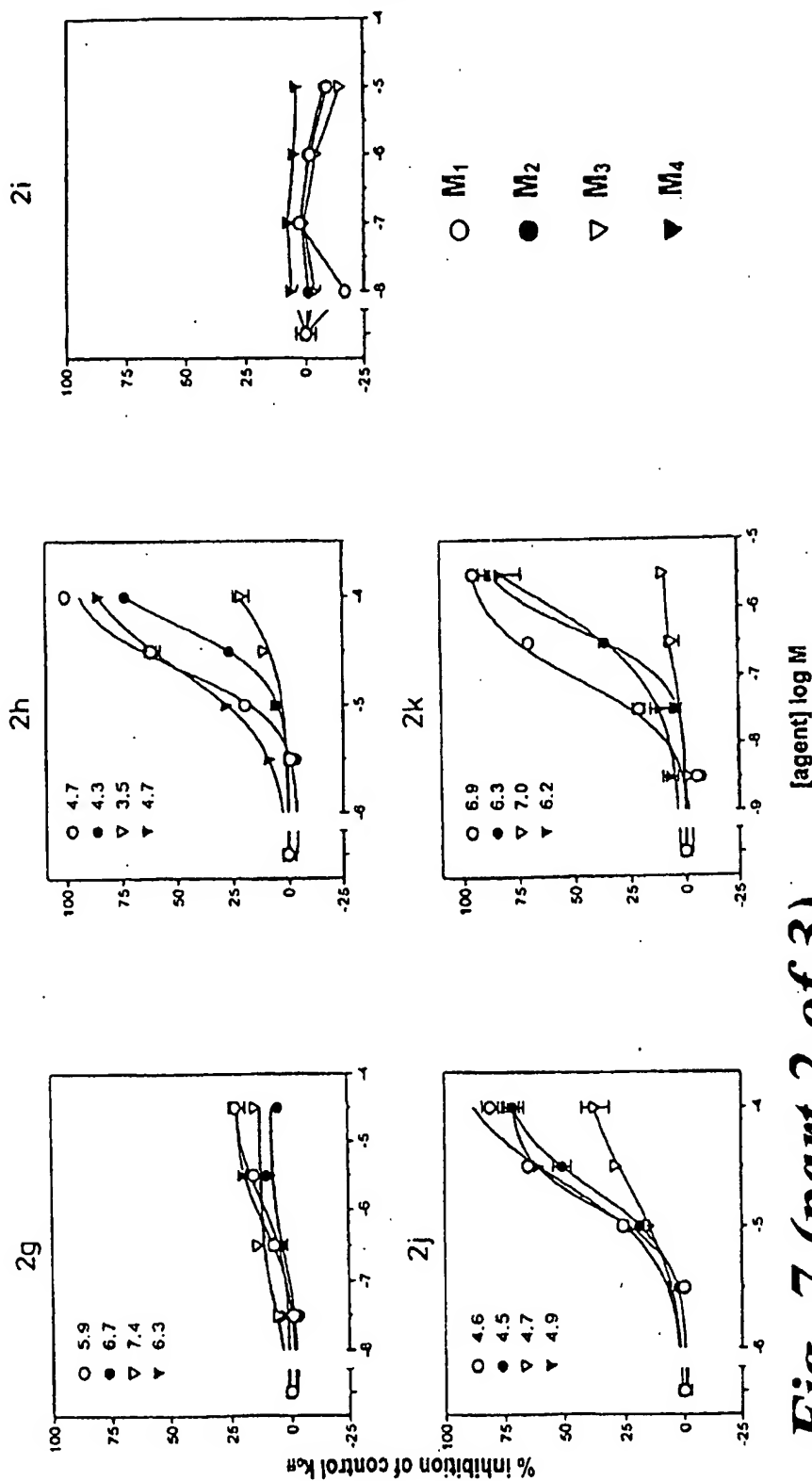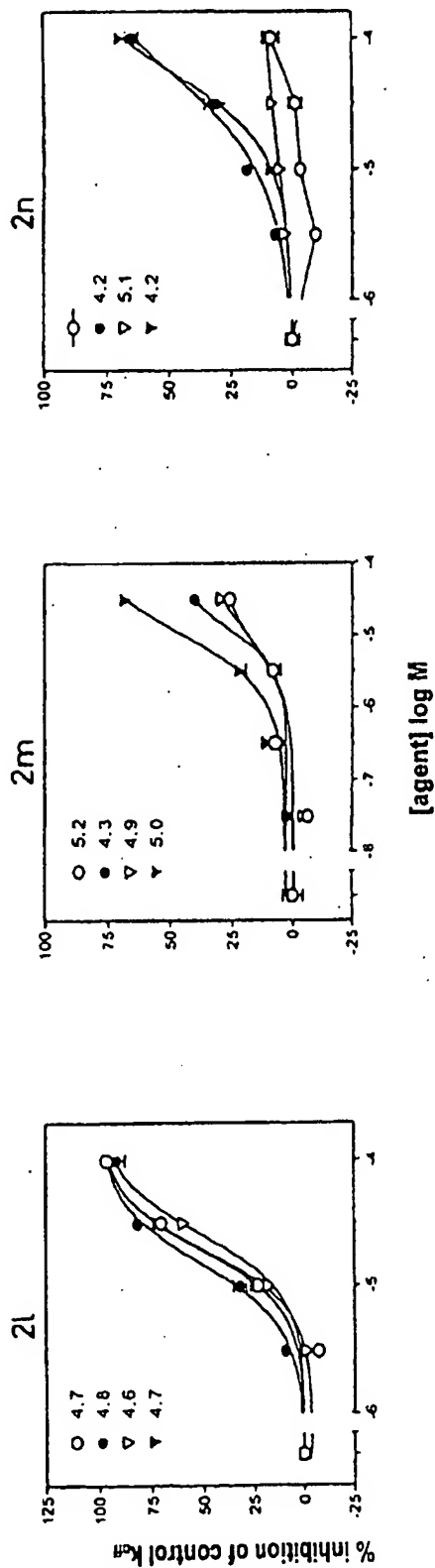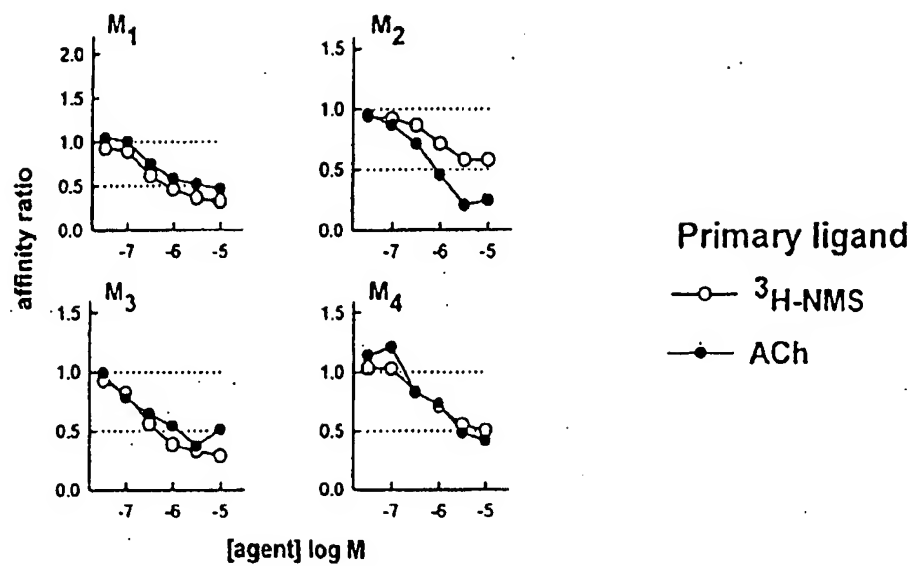
2b(WIN 51708)

Fig. 8 (part 1 of 3)

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Fig. 8 (part 2 of 3)

Fig. 8 (part 3 of 3)

Fig. 9 (part 1 of 3)

Fig. 9 (part 2 of 3)

Fig. 9 (part 3 of 3)

Fig. 10

Fig. 11 (part 1 of 2)

Fig. 11 (part 2 of 2)

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Fig. 12 (part 1 of 2)

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Fig. 12 (part 2 of 2)

(54) Title: ALLOSTERIC SITES ON MUSCARINIC RECEPTORS

(57) Abstract: An allosteric site on muscarinic receptors is disclosed, together with its use for screening for compounds capable of modulating the binding of a primary ligand such as acetylcholine to the receptor. The site is characterised herein a series of indolocarbazoles represented by formula (1) and a series of related compounds represented by formula (2). These compounds are capable of binding to the allosteric site to modulate the binding of a primary ligand to the receptors, showing positive, negative and neutral cooperativity and selectivity for muscarinic receptor subtypes.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   G01N33/566   A61K31/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   G01N   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 292 685 A (SANKYO CO) 6 March 1996 (1996-03-06) cited in the application abstract ---	1-16,29
X	WO 96 03377 A (BIRDSALL NIGEL ;LAZARENO SEBASTIAN (GB); SUGIMOTO MASAHIKO (JP); N) 8 February 1996 (1996-02-08) cited in the application abstract --- -/--	1-16,29

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2001	12 07 2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hart-Davis, J

Form PCT/ISA/210 (second sheet) (July 1992)

Inter onal Application No

PCT/GB 00/04064

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIRDSALL NIGEL J M ET AL: "Subtype-selective positive cooperative interactions between brucine analogs and acetylcholine at muscarinic receptors: Functional studies." MOLECULAR PHARMACOLOGY, vol. 55, no. 4, April 1999 (1999-04), pages 778-787, XP000997267 ISSN: 0026-895X cited in the application the whole document ---	1-16,29
X	LAZARENO S ET AL: "Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: Radioligand binding studies." MOLECULAR PHARMACOLOGY, vol. 53, no. 3, March 1998 (1998-03), pages 573-589, XP000997226 ISSN: 0026-895X cited in the application the whole document ---	1-16,29
X	LAZARENO SEBASTIAN ET AL: "Detection, quantitation and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: Interactions of strychnine and acetylcholine at muscarinic receptors." MOLECULAR PHARMACOLOGY, vol. 48, no. 2, 1995, pages 362-378, XP000997227 ISSN: 0026-895X cited in the application the whole document ---	1-16,29
X	LAZARENO S ET AL: "Allosteric effects of four stereoisomers of a fused indole ring system with 3H-N-methylscopolamine and acetylcholine at M1-M4 muscarinic receptors." LIFE SCIENCES, vol. 64, no. 6-7, 8 January 1999 (1999-01-08), pages 519-526, XP000997312 Eighth International Symposium on Subtypes of Muscarinic Receptors;Danvers, Massachusetts, USA; August 25-29, 1998 ISSN: 0024-3205 the whole document ---	1-16,29

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Inte   'onal Application No

PCT/GB 00/04064

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LAZARENO SEBASTIAN ET AL: "Allosteric interactions of staurosporine and other indolocarbazoles with N-(methyl-3H)scopolamine and acetylcholine at muscarinic receptor subtypes: Identification of a second allosteric site." MOLECULAR PHARMACOLOGY, vol. 58, no. 1, July 2000 (2000-07), pages 194-206, XP000997198 ISSN: 0026-895X the whole document ---	1-16,29
A	US 5 747 336 A (BUCKLEY NOEL J ET AL) 5 May 1998 (1998-05-05) column 5, line 22 - line 64; table 1 ---	1-16,29
A	US 5 677 327 A (WOLDEMUSSIE ELIZABETH ET AL) 14 October 1997 (1997-10-14) claim 1 ---	1-16,29
A	WO 96 13506 A (KYOWA HAKKO KOGYO KK ;CEPHALON INC (US)) 9 May 1996 (1996-05-09) abstract page 2, line 8 - line 15 ---	
A	US 5 658 898 A (WEDER HANS GEORG ET AL) 19 August 1997 (1997-08-19) abstract column 1, line 33 - line 40 -----	

1

INTERNATIONAL SEARCH REPORT

| Box I | Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet) |

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II | Observations where unity of invention is lacking (Continuation of item 2 of first sheet) |

This International Searching Authority found multiple inventions in this International application, as follows:

> see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

> see further information sheet inevention 1

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

This International Searching Authority found multiple (groups of)
inventions in this international application, as follows:

1. Claims: 1-16, 29

> Methods for identifying compounds capable of modulating the
> binding of a primary ligand to a muscarinic receptor by
> binding to an allosteric site of the muscarinic receptor.

2. Claims: 17-28, 30-31 (all partially)

> Compounds for medical treatment, wherein the compounds are
> represented by the formulae 1a, 1b, 1c, 1d and 1e (page 55).

3. Claims: 17-28, 30-31 (all partially)

> Compounds for medical treatment, wherein the compounds are
> represented by the formula 1f (page 55).

4. Claims: 17-28, 30-31 (all partially)

> Compounds for medical treatment, wherein the compounds are
> represented by the formulae 1g, 1h and 1i (page 55).

5. Claims: 17-28, 30-31 (all partially)

> Compounds for medical treatment, wherein the compounds are
> represented by the formulae 2a, 2b, 2c, 2d, 2e, 2f, 2g, 2h,
> 2i, 2j, 21, 2u (pages 56-57; see also general formula of
> page 4).

6. Claims: 17-20, 22-28 (all partially)

> Compounds for medical treatment, wherein the compounds are
> represented by the formulae 2k, 2m, 2n, 2o, 2p, 2q or 2r
> (pages 56-57).

7. Claims: 17-20, 22-28 (all partially)

> Compounds for medical treatment, wherein the compounds are
> represented by the formula 2s (page 57).

8. Claims: 17-20, 22-28 (all partially)

> Compounds for medical treatment, wherein the compounds are
> represented by the formula 2t (page 57).

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

INTERNATIONAL SEARCH REPORT

.nformation on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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